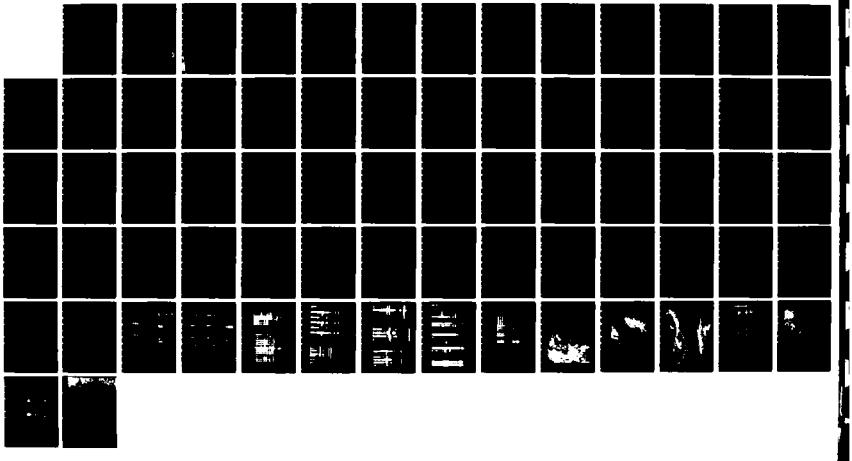
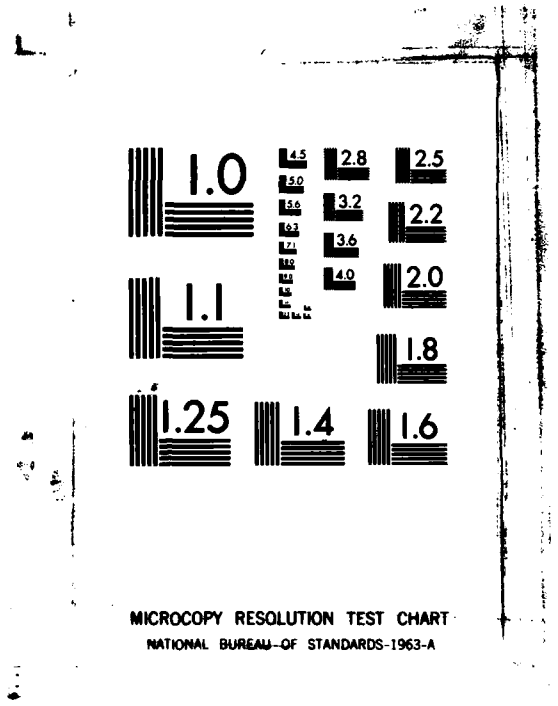
AD-A140 147 IN VITRO STUDIES OF SANDFLY FEVER VIRUSES AND THEIR POTENTIAL SIGNIFICANC..(U) MARYLAND UNIV BALTIMORE DEPT OF MICROBIOLOGY J F SMITH FEB 80 DAMD17-78-C-8056 1/1

UNCLASSIFIED F/G 6/5 NL





AD _____

In vitro Studies of Sandfly Fever Viruses and Their Potential Significance for Vaccine Development

Annual Progress Report

by

Jonathan F. Smith, Ph.D.

February 1980

(for the period 1 January 1979 - 1 January 1980)

Supported by

U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
Fort Detrick, Frederick, Maryland 21701

Contract No. DAMD-17-78-C-8056

University of Maryland School of Medicine
Department of Microbiology
Baltimore, Maryland 21201

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

REPORT DOCUMENTATION PAGE

READ INSTRUCTIONS BEFORE COMPLETING FORM

1. REPORT NUMBER: DAMD-17-78-C-8056	2. GOVT ACCESSION NO.: AD-A140 147
3. RECIPIENT'S CATALOG NUMBER:	
4. TITLE (and Subtitle): In vitro Studies of Sandfly Fever Viruses and Their Potential Significance for Vaccine Development	5. TYPE OF REPORT & PERIOD COVERED: Annual Report 1 January 1979-1 January 1980
	6. PERFORMING ORG. REPORT NUMBER:
7. AUTHOR(s): Jonathan F. Smith, Ph.D.	8. CONTRACT OR GRANT NUMBER(s): DAMD-17-78-C-8056
9. PERFORMING ORGANIZATION NAME AND ADDRESS: University of Maryland Medical School, Department of Microbiology, 660 W. Redwood St., Baltimore, Md. 21201	10. PROGRAM ELEMENT, PROJECT, TASK AREA & WORK UNIT NUMBERS: 61102A.3M161102BS10.AA.074
11. CONTROLLING OFFICE NAME AND ADDRESS: U.S. Army Medical Research & Development Command, Fort Detrick, Frederick, Maryland 21701	12. REPORT DATE: February 1980
	13. NUMBER OF PAGES: 69
14. MONITORING AGENCY NAME & ADDRESS (if different from Controlling Office)	15. SECURITY CLASS. (of this report): Unclassified
	15a. DECLASSIFICATION/DOWNGRADING SCHEDULE

16. DISTRIBUTION STATEMENT (of this Report)
Approved for public release; distribution unlimited.

17. DISTRIBUTION STATEMENT (of the abstract entered in Block 20, if different from Report)

18. SUPPLEMENTARY NOTES

19. KEY WORDS (Continue on reverse side if necessary and identify by block number)
Sandfly Fever, Phlebotomus Fever, Viruses, Arboviruses, replication, bunyaviridae, vaccines, structural proteins, non-structural proteins, processing, glycopeptides, glycosylation, morphogenesis, maturation, transmembranal proteins, antigen purification, immunoprecipitation, gel electrophoresis, neutralization Karimibad virus, Punta Toro virus

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)
Several aspects of the replication of sandfly fever viruses have been examined in BHK or vero cell cultures. We have expanded the immunoprecipitation techniques developed in the preceding year to identify structural proteins, glycoproteins, and putative non-structural proteins of several sandfly fever viruses. The results have indicated that overall similarities in the profiles of virus-specific proteins exist, but that each virus directs the synthesis of a set of proteins which is recognizably different from other viruses. Several polypeptides have been identified in lysates prepared from cells infected with sandfly fever viruses

DD FORM 1 JAN 73 1473 EDITION OF 1 NOV 65 IS OBSOLETE

SECURITY CLASSIFICATION OF THIS PAGE (When Data Entered)

20. (Continued)

which may be termed non-structural insofar as they do not become in integral part of the mature virion, and this represents the first description of such proteins among viruses in the family, Bunyaviridae. Although it is probable, it remains to be shown that putative non-structural proteins are, in fact, unique gene products. The kinetics of synthesis of individual Karimibad virus-specific proteins has been studied, and it has been shown that controls exist which effect both the temporal and quantitative production of viral proteins.

Several experiments have examined the post-translational processing of proteins specified by Karimibad and Punta Toro viruses. These studies have suggested that proteolytic cleavages occur in the processing of glycoproteins and perhaps other proteins in their conversion to mature viral proteins, probably as co-translational events since they are only demonstrable in the presence of protease inhibitors. Experiments using zinc ions to inhibit proteolysis of primary gene products have provided preliminary evidence that high molecular weight polyprotein precursors may also be synthesized in virus-infected cells, which if confirmed, would indicate that messenger RNA synthesis occurs at least in part, polycistronically. Post-translational glycosylation of the viral membrane proteins has also been studied, and the size and structure of Punta Toro and Karimibad virus glycopeptides has been determined. Glycosylation of all viral glycoproteins occurs exclusively at asparagine residues, is limited to small, polymannose-type oligosaccharides, and consists entirely of N-acetylglucosamine and mannose. Among the many enveloped viruses which have been studied, this pattern is apparently unique to sandfly fever viruses, is probably a function of internal maturation at Golgi membranes, and should be useful in the purification of virus particles and viral antigens by lectin-affinity techniques.

As monitored by ferritin-tagged antibodies and electron microscopy, it will be shown that the maturation and release of these viruses does not result in appreciable modification of host cell plasma membranes with viral antigens. It has also been shown that the release of virus is not inhibited by the presence of specific anti-virus antibody.

The role of the nucleus with respect to virus replication has been studied in light of the knowledge that viral antigen appears to be present within these structures and that at least one bunyavirus, Bunyamwera, fails to replicate in enucleated cells. Our studies have shown that intranuclear antigen is apparently limited to the nucleocapsid protein, that unlike the orthomyxoviruses, viral antigens do not accumulate in nuclei in the presence of amino acid analogues, and that transcription of host cell DNA is not required for the synthesis of viral mRNA or protein. Consequently, the manner in which DNA-specific transcription inhibitors and enucleation partially inhibit viral replication remains unknown.

The processes which occur during virion assembly have been studied by the analysis of membranes isolated from infected cells. Data will be presented to demonstrate that all structural proteins, but not all non-structural proteins, become membrane-bound. Resistance to protease digestion has shown that membrane-bound viral proteins differ with respect to their relative position on these membranes, and that at least one, and perhaps both viral glycoproteins are transmembranal during virus budding, and probably remain so in the mature virion. The significance of these observations is discussed, both here and in the accompanying proposal, with respect to the events which take place during virus maturation and the roles of specific proteins in this process.

20. (Continued)

Reciprocal cross-immunoprecipitations have been carried out using lysates prepared from Karimbad and Punta Toro virus-infected cells and homologous and heterologous antisera. This experiment has demonstrated that shared antigenic determinants can be traced to individual viral proteins by these procedures. Furthermore these data suggest that the determinants which are responsible for hemagglutination and virus neutralization reside on different surface proteins.

Table of Contents

I. Abstract

Several aspects of the replication of sandfly fever viruses have been examined in BHK or vero cell cultures. We have expanded the immunoprecipitation techniques developed in the preceding year to identify structural proteins, glycoproteins, and putative non-structural proteins of several sandfly fever viruses. The results have indicated that overall similarities in the profiles of virus-specific proteins exist, but that each virus directs the synthesis of a set of proteins which is recognizably different from other viruses. Several polypeptides have been identified in lysates prepared from cells infected with sandfly fever viruses which may be termed non-structural insofar as they do not become an integral part of the mature virion, and this represents the first description of such proteins among viruses in the family, Bunyaviridae. Although it is probable, it remains to be shown that putative non-structural proteins are, in fact, unique gene products. The kinetics of synthesis of individual Karimibad virus-specific proteins has been studied, and it has been shown that controls exist which effect both the temporal and quantitative production of viral proteins.

Several experiments have examined the post-translational processing of proteins specified by Karimibad and Punta Toro viruses. These studies have suggested that proteolytic cleavages occur in the processing of glycoproteins and perhaps other proteins in their conversion to mature viral proteins, probably as co-translational events since they are only demonstrable in the presence of protease inhibitors. Experiments using zinc ions to inhibit proteolysis of primary gene products have provided preliminary evidence that high molecular weight polyprotein precursors may also be synthesized in virus-infected cells, which if confirmed, would indicate that messenger RNA synthesis occurs at least in part, polycistronically. Post-translational glycosylation of the viral membrane proteins has also been studied, and the size and structure of Punta Toro and Karimibad virus glycopeptides has been determined. Glycosylation of all viral glycoproteins occurs exclusively at asparagine residues, is limited to small, polymannose-type oligosaccharides, and consists entirely of N-acetylglucosamine and mannose. Among the many enveloped viruses which have been studied, this pattern is apparently unique to sandfly fever viruses, is probably a function of internal maturation at Golgi membranes, and should be useful in the purification of virus particles and viral antigens by lectin-affinity techniques.

As monitored by ferritin-tagged antibodies and electron microscopy, it will be shown that the maturation and release of these viruses does not result in appreciable modification of host cell plasma membranes with viral antigens. It has also been shown that the release of virus is not inhibited by the presence of specific anti-virus antibody.

The role of the nucleus with respect to virus replication has been studied in light of the knowledge that viral antigen appears to be present within these structures and that at least one bunyavirus, Bunyamwera, fails to replicate in enucleated cells. Our studies have shown that intranuclear antigen is apparently limited to the nucleocapsid protein, that unlike the orthomyxoviruses, viral antigens do not accumulate in nuclei in the presence of amino

acid analogues, and that transcription of host cell DNA is not required for the synthesis of viral mRNA or protein. Consequently, the manner in which DNA-specific transcription inhibitors and enucleation partially inhibit viral replication remains unknown.

The processes which occur during virion assembly have been studied by the analysis of membranes isolated from infected cells. Data will be presented to demonstrate that all structural proteins, but not all non-structural proteins, become membrane-bound. Resistance to protease digestion has shown that membrane-bound viral proteins differ with respect to their relative position on these membranes, and that at least one, and perhaps both viral glycoproteins are transmembranal during virus budding, and probably remain so in the mature virion. The significance of these observations is discussed, both here and in the accompanying proposal, with respect to the events which take place during virus maturation and the roles of specific proteins in this process.

Reciprocal cross-immunoprecipitations have been carried out using lysates prepared from Karimibad and Punta Toro virus-infected cells and homologous and heterologous antisera. This experiment has demonstrated that shared antigenic determinants can be traced to individual viral proteins by these procedures. Furthermore, these data suggest that the determinants which are responsible for hemagglutination and virus neutralization reside on different surface proteins.

II. General Introduction

Among the approximately 400 viruses which are presently classified as arboviruses, about 160 belong to the family Bunyaviridae or are classified as "bunyavirus-like", and approximately one-fourth of these are known to cause significant human disease. Thus, collectively, bunyaviruses constitute the largest organized family of arboviruses, and the largest group among Bunyaviridae, the phlebotomus or sandfly fever group, will be the subject of this report. Despite their importance as human and animal pathogens, the Bunyaviridae remain the least well understood of the major arbovirus group in terms of their basic virology and replication patterns which they establish in infected cells. On the surface there would appear to be several reasons for this, which are not necessarily interrelated or related to the disease potential of this group of viruses.

First, many alphaviruses and rhabdoviruses, and to a lesser extent flaviviruses, have been chosen as model systems to study diverse aspects of animal viruses or eukaryotic processes in general. These aspects include RNA replication, RNA transcription, membrane biogenesis, protein glycosylation, induction and action of interferon, and formation of defective particles. Second, alphaviruses and rhabdoviruses replicate rapidly in a wide variety of cell types, producing high titers of virus which can be easily assayed and purified. Bunyaviruses, on the other hand, generally produce lower titers of virus which, in addition, tend to be unstable during standard concentration and purification methods, and many are difficult to assay by standard plaque assay procedures. Thus, they are less attractive as model systems to investigators who are less interested in the virology of these agents, per se.

For these reasons and others, the Bunyaviridae have only recently been studied from the point of view of molecular virology and consequently the information base is limited compared to other arbovirus groups. This is not, however, a reflection of their relative medical importance. The California encephalitis group, for example, is responsible for a significant percentage of arbovirus-induced encephalitis in North America, and Rift Valley Fever and Congo-Crimean hemorrhagic fever are associated with significant mortality rates. Many other bunyaviruses such as the sandfly fever viruses, while causing non-fatal infections, are acute, incapacitating diseases with complications persisting after defervescence. Recent serological evidence has now shown that Rift Valley Fever (RFV) virus properly belongs in the sandfly fever group (2), indicating that at least one, and perhaps other members of this group, may induce more serious illness than has been associated with the more classical members of the group such as the Naples and Sicilian strains.

In addition, the epidemiology of these diseases (also reviewed in accompanying report) is such that explosive epidemics can occur following either 1) the introduction of non-immune individuals into endemic areas, or 2) the introduction of exogenous strains or strains of increased virulence into areas previously free of such viruses. For the former reason sandfly fever has historically been of considerable military importance (see Section IV, renewal proposal), and the recent RVF epidemic in Egypt (an area in which RVF was previously unknown), in which a minimum of 18,000 cases and 600 deaths occurred,

adequately illustrates the latter. It is also noteworthy that, while not limited to these areas, the geographical occurrence of sandfly fever from North Africa east to Pakistan and India, of Rift Valley fever from central to North Africa, and of Congo-Crimean hemorrhagic fever from central Africa through the mid-east and into south-central Asia, currently incudes areas of some strategic importance.

Aside from the serological studies which have established groups and subgroups of Bunyaviridae, most of what is known about this family of viruses has come from studies examining 1) the composition of isolated virions, 2) the morphology and morphogenesis of viruses by electron microscopy, and 3) the genetics of conditionally lethal (ts) mutants (all of which are reviewed and referenced in the renewal proposal). Essentially, nothing is known about the synthesis and processing of viral RNA and proteins during the replication of these viruses in permissive host cells. The conclusions which have emerged from the studies listed above have shown that the Bunyaviridae constitute a unique group of viruses with a set of basic structural and morphogenic characteristics which are not shared by any other group of viruses (see reference 3 for recent review).

The genomic RNA is single-stranded, negative-stranded, and segmented into three size classes, each of which contains unique nucleotide sequences. These RNA segments are complexed with the smallest structural protein (N) and presumably also a virion-associated transcriptase which is required due to the negative sense of the genome. Both the RNA and the ribonucleoprotein (RNP) can be isolated as closed circular structures although the presence of 3' and 5' ends of the RNA demonstrates that the RNA is not covalently circularized. The ribonucleoprotein (RNP) is helically structured and packaged within an enveloped virion which is roughly spherical in shape and approximately 1000 A in diameter.

There are two virus-coded glycoproteins associated with the envelope which vary in molecular weight in different bunyaviruses. There is apparently no M-type protein which is believed to form a bridge between the viral RNP and spike glycoproteins of rhabdoviruses, influenza viruses, parainfluenza viruses and RNA tumor viruses. This suggests that one or both of the virion glycoproteins may be transmembranal (see Section VI- 3). An M-type protein is also lacking in the arenaviruses which bear other (at least superficial) resemblances to bunyaviruses. These include a segmented, negative-stranded genome, a similar assortment of proteins, and a lipid-containing envelope. However, unlike the arenaviruses which mature at the plasma membrane, the bunyaviruses bud into smooth internal membrane structures, at least some of which are Golgi lamellae. The internal maturation of these viruses appears to be unique among negative-stranded RNA viruses. These structural and morphogenic features of bunyaviruses therefore distinguish the family Bunyaviridae from other groups of animal viruses and suggest that the extrapolation of data, conclusions, or expected experimental results from other systems is unwarranted.

Genetic studies have shown that during envelopment of these viruses, progeny RNP segments from different infecting viruses can be reassorted forming recombinant viruses (3, 4). From such cloned recombinant viruses, in which it has been possible to identify the parental source of the RNA and proteins contained within the particle, it has been possible to show that the smallest of the genomic RNA segments specifies the N protein, and that the medium-sized segment codes for both glycoproteins. However, it has not been possible to show recombination between bunyaviruses from different serological groupings, and not all crosses among the same group have been successful. From a virus containing three genome segments it would expected a priori that three recombination groups would exist generating six new genotypes in two-factor crosses. However, only two recombination groups and restricted recombination have so far been demonstrated. One possible interpretation of this data is that not all bunyaviruses have equivalent functions encoded in equivalent segments; another is that the viral proteins involved in these functions are, to at least some degree, strain-specific. (That the latter may be the case will be shown within the body of this report where we will show that significant differences exist not only in structural proteins but also in putative non-structural proteins).

As has been shown by us (1979 annual report) and by others (1, 5), the sandfly or phlebotomus fever viruses possess virion RNA, virion proteins, and morphogenesis characteristics which firmly place this group within the family Bunyaviridae. This group presently contains 30 serologically distinct viruses, at least six of which areknown to be human pathogens. Five of these pathogens, Naples and Sicilian sandfly fever, Punta Toro, Chagres, and Candiru produce a non-fatal, but acute, incapacitating febrile illness with mental depression and other symptoms persisting in some cases for several weeks. The sixth sandfly fever group virus associated with human disease, Rift Valley fever virus, produces a more serious syndrome. In the recent Egyptian epidemic the disease presented mainly as a dengue-like illness but with many cases showing hemorrhagic complications, meningo-encephalitis, and degenerative retinitis; 598 deaths were reported among 18,000 cases (6). Therefore, although it is unclear why the Egyptian RVF isolate seemed to be more virulent than strains isolated from neighboring areas in previous epidemics, the clinical response to infection by this and other phlebotomus group viruses has been characterized.

Similarly, as documented in the 1978 proposal and summarized in the current proposal, the epidemiology and vector relationships of the classical sandfly fever agents has been well studied. These agents are vectored by Phlebotomus or Lutzomyia, and transovarial transmission is believed to allow persistence in endemic areas irrespective of the immune status of the indigenous population and in the absence of animal reservoirs. Rift Valley fever virus has been isolated from mosquito pools of Culex pipiens(although it seems clear that this is not the only possible vector, 2,6), and the association of this virus with domestic animals and animal products has long been recognized.

Control of these diseases resides in vector eradication, and potentially in vaccines and antivirals. Sabin demonstrated as early as 1944 that both the Sicilian and Naples strains could be attenuated by mouse brain passage and that such brain preparations could be successfully used as vaccines insofar as vaccinated individuals did not develop clinical disease and were resistant to experimental challenge with the homologous agent (29, 30). Sabin's studies show quite conclusively that protection from these diseases by vaccination is a realistic goal, although these mouse braine preparations are no longer acceptable due to potential neuroparalytic complications. The challenge at present is to produce additional vaccines in accordance with current knowledge and regulations relating to safety and efficacy. The data which is presently lacking, which will complement the studies referred to above, and which will be necessary to design, produce, and test experimental immunogens or determine the action of antiviral agents, relates to the basic virology of the phlebotomus agents. Data are needed on the intracellular replication of these viruses, on the logistical problems of preparing and purifying virus grown in cell substrates acceptable for vaccine production, on the controls imposed upon virus gene expression, and on the synthesis and function of virus-coded proteins and antigens - both in terms of their role in virion maturation and as antigens which potentially could offer immunological protection.

As indicated in the previous proposal, the central points which have been addressed in our studies with phlebotomus viruses are:

1) to identify virus-coded structural and non-structural proteins

2) to characterize the post-translational processing events which are carried out on these proteins

3) to determine the "strategy" of the genome, i.e., the manner in which expression of the viral genome is controlled

4) to characterize the morphogenic patterns of these agents and the intracellular localization of viral antigens

5) to test the protective effect of native or chemically altered virion glycoproteins in a mouse model.

Since the structure and morphogenesis of all bunyaviruses which have been studied seems similar, it is hoped that at least the general conclusions of these studies of sandfly fever replication could be extended to other bunyaviruses.

Most of these objectives are clearly interrelated and are studied as such. For example, the manner in which genome expression is controlled will determine at least some of the post-translational processing events which occur. Since there are more virus-specific proteins than genome segments (even without consideration of non-structural proteins), either polycistronic messengers result in the production of precursor polyproteins which are cleaved during the post-translational processing, or multiple but monocistronic messengers originate from single genomic polynucleotides (see Discussion in Section III-B, renewal proposal). Thus, the demonstration of a non-structural, precursor protein containing sequences of mature proteins would argue very strongly for polycistronic transcription.

In the preceding annual report we showed very preliminary data in which immunoprecipitation techniques were used to identify Karimibad and Punto Toro virus proteins from infected cells. Both structural and apparently non-structural proteins were demonstrated. We have now confirmed the identification of structural proteins and the existence of putative non-structural proteins, and have extended these studies to several other sandfly viruses. In addition, these techniques have now been used to monitor the kinetics of synthesis of individual viral proteins, to suggest that proteolytic cleavages are involved in the processing of at least the envelope glycoproteins and probably other proteins, and to show that at least one of the glycoproteins is transmembranal at least during morphogenesis. Using immune selection we have analyzed the glycopeptide and glycosylation patterns of the virus-specific glycoproteins. We will present data to show that such patterns have not been found in other viruses, and that they may be useful in the purification of viruses or viral antigens. Immunoprecipitation techniques have also allowed us to assess the effect of DNA-specific transcription inhibitors on viral protein synthesis, and thus to demonstrate that (unlike orthomyxoviruses) viral mRNA synthesis is independent of host DNA transcription. Preliminary results will also be presented to indicate that UV irradiation of virus particles prior to infection is a useful technique not only to identify virus-specific proteins synthesized in infected cells, but also to study the manner in which viral RNA transcription is controlled. Other studies relative to the distribution of viral antigens in infected cells are now completed and will be discussed. Thus, several fundamental aspects of sandfly fever viruses and their intracellular replication have been found to be approachable by these immunoprecipitation procedures, and as a result, this report and the accompanying proposal will consequently be heavily weighted in favor of these in vitro studies. The evaluation of the in vivo immunogenicity of viral envelope antigens (see 1979 proposal) has been hindered by the recognized instability of these viruses during purification (3, 8, 81, 83). Alternative approaches of antigen purification are discussed both here and in the accompanying proposal.

III. Procedures

1) Discussion of Immuno-selection Procedures and the Intepretation of Results

Since much of the data which will be presented in this report has been collected in experiments in which immunoprecipitation has been used to detect virus-specific polypeptides, it is perhaps worthwhile to consider the advantages and disadvantages of the various methodologies available and the problems involved in the interpretation of this type of data. In RNA virus-host systems in which the host-specific synthesis is inefficiently terminated after infection, and in which the efficiency of virus-specific protein synthesis is significantly reduced in the presence of DNA-specific transcription inhibitors, the assigning of proteins synthesized after infection as either virus or host-coded has been difficult. Such problems are compounded if it is not possible to show a relationship to a known virion structural protein.

With Bunyaviridae these problems are illustrated by the reported studies of David West (9) who was able to demonstrate only one protein thought to be virus-specific in infected cell lysates and by the recent studies of Lasdins and Holmes (7) and Vezza, et al. (10) who were unable to demonstrate consistently even the known structural proteins of the viruses under study. Since the combined molecular weight of the genome segments is much larger than that required to code for the structural proteins (including a high molecular weight transcriptase), and the nucleotide sequence of each segment studied has been shown to be unique, it would be surprising if the presently recognized structural proteins constituted the only proteins specified by these viruses.

In our studies of sandfly fever viruses we initially attempted to examine viral protein synthesis by direct isotope incorporation into infected cells which had been inhibited by either actinomycin D or DRB (5, 6-Dichloro-1-β-Ribofuranosylbenzimidazole) - both inhibitors of DNA-dependent RNA synthesis (58). These studies were unsuccessful due to both residual host-specific protein synthesis and a depression of viral functions. We have therefore chosen to select and concentrate viral proteins by immunoprecipitation. We have shown in the previous report and in studies which are extended here that virion structural polypeptides as well as apparent non-structural proteins, can be detected by this procedure from lysates of infected cells. The source of the antibodies used in these experiments was hyperimmune mouse ascitic fluid (HMAF) raised in response to the injection of infected suckling mouse brain homogenates (obtained as a gift from Dr. Walter Brandt, WRAIR). Such preparations were adsorbed with uninfected cells and should contain activity against all virus-coded polypeptides although this remains an assumption which is difficult to test at this time. The experimental evidence that suggests viral specificity of these polypeptides is the following:

1) Polypeptides are demonstrable only after infection (i.e., not from uninfected cells).

2) Polypeptides can be consistently demonstrated in infected but unrelated host cells (vero and BHK).

3) Polypeptides are not precipitated by normal mouse ascitic fluids (NMAF).

4) Immunoprecipitation of a mixture composed of lysates from labelled uninfected cells and unlabelled infected cells do not show these polypeptides.

Although it is likely that proteins which satisfy these criteria are virus-specified, these criteria do not provide rigorous proof, nor should it be assumed that all such polypeptides, even if virus-specific, are unique. Some polypeptides may be related to others by precursor-product relationships or they may be mistranslation products or processing failures. The "B" protein found in Sindbis virus-infected cells, for example, contains sequences of both E_1 and PE_2. However, the "B" protein accumulates in infected cells, it is not cleaved, and it is not glycosylated (11). Perhaps cleavage of this protein must occur co-translationally to be inserted into the membrane of the endoplasmic reticulum, or perhaps insertion is required for cleavage. Nevertheless, the occurrence of this protein

illustrates the point that metabolic instability is insufficient evidence for a unique gene product. Another problem exists when virus infection does not adequately terminate host-specific synthesis, but may alter it either quantitatively or qualitatively as occurs in cells infected with many different viruses. Virus infection could induce host proteins or arrest infected cells at a particular point in the cell cycle when a specific subset of cell proteins are produced. Any such alteration in the host cell polypeptides which are in addition cross reactive with the antibody used could appear to be virus-induced since uninfected cells would cease to be valid controls.

Additional problems relate directly to the techniques which are available. The solubilization of membrane proteins requires the presence of detergents which essentially replace bound membrane lipids, and hence, disrupt membranes and render amphipathic proteins soluble. Strongly ionic detergents which are the most efficient at solubilization (e.g., sodium dodecyl sulfate) are unfortunately incompatible with antibody function. The advantages of non-ionic detergents lie in their moderate solubilizing ability without causing protein denaturation. This, however, leaves open the possibility that some membranes may be incompletely solubilized (the inner nuclear membrane, for example, remains intact in the presence of triton X-100), and incomplete solubilization may co-precipitate (cell) proteins which are actually not recognized by the antibody directly. A related problem occurs when proteins which have been adequately solubilized from membranes form mixed micelles with the detergent which then contain unrelated proteins - again resulting in co-precipitation of unreacting antigens. Finally, "sticky" proteins have been noted, which, for unknown reasons, have an affinity for antigen-antibody complexes.

In attempts to control for these problems, we have used a mixture of detergents (1% triton X-100 and 0.5% sodium deoxycholate) which maximizes membrane solubilization and minimizes mixed-micelle formation. The fact that we have obtained distinct bands after isoelectric focusing with these concentrations of detergents indicates that incomplete membrane solubilization and mixed micelle formation are not serious problems in our experiments. We have attempted to control against co-precipitation of so-called "sticky" proteins by the procedure outlined above (number 4), and for the remainder of these problems, by analyzing uninfected cells in parallel with infected cells. However, fluorographs are intentially overexposed to reveal minor polypeptide species - thus increasing the background, and it is recognized that if host proteins are concentrated in immunoprecipitates for any of the other reasons discussed above, misleading results could be obtained.

Unrelated evidence that the putative non-structural proteins are, in fact, virus-specific, has come from our preliminary studies on transcriptional controls in which virus is irradiated with ultraviolet light prior to infection. With increasing doses of UV light the synthesis of these polypeptide species is decreased. These data will be discussed below. However, rigorous proof of viral specificity must await either 1) in vitro translation experiments, 2) the demonstration of tryptic peptide analysis of relationships with known virus structural proteins, or 3) studies of temperature-sensitive mutants. Such studies will be carried out in the following year and will yield information on other aspects of viral replication (see renewal application). Although it is important to recognize that immunoprecipitation techniques possess several inherent problems,

these techniques have allowed us to approach problems (reviewed in this report) which cannot be addressed by any other means. Also, these methods are experimentally convenient insofar as only very small quantities of reagents and cells are necessary and many samples can be analyzed concurrently. In my opinion, the data collected from several sandfly virus systems are sufficiently strong to warrant the continued study of virus replication and proetin processing by these techniques.

2) Specific Methodology

A. Immunoprecipitation

Subconfluent monolayers of either BHK or vero cells were infected with sandfly fever viruses at a multiplicity of infection of 0.1 to 10 (as indicated). At appropriate times (usually 14-18 hrs) after infection the cells are labelled with 50 microcuries of tritiated leucine per ml in leucine-free media or 200 microcuries per ml of 2-^{3}H-mannose. At the end of the labelling period the cells are removed from the surface and lysed in buffer containing 1% triton X-100, 0.02M tris-HCl buffer (pH 7.5), 0.05 m NaCl and 5 micromolar PMSF (phenylmethane sulphonyl fluoride, protease inhibitor). The cells are incubated in this buffer for 15 minutes at 4^{o}C, gently homogenized, and the nuclei (except where noted) are removed by centrifugation, DOC is then added to a final concentration of 0.5% and the lysate is centrifuged for 2 mintues at 15,000 RPM in a Brinkman microfuge. Viral proteins can then be selected from this lysate by either 1) direct precipitation, 2) indirect precipitation using staphylococcal protein A, or 3) immunoaffinity chromatograpy. Immuno-affinity chromatography, in which antibody is covalently linked to agarose beads (see 1979 annual report) has been used for preparative procedures, but is not practical for the analysis of large numbers of samples.

Direct precipitation has been found to give the lowest background of host cell polypeptides, but various dilutions of HMAF must be added to each lysate sample to assure equivalence point precipitation, thus limiting the number of samples which can be analyzed and increasing greatly the amount of immunoglobulin required. We have found that indirect precipitation using protein A (protein A-sepharose, Pharmacia; or Pansorbin, Calbiochem) is the most efficient of the three procedures in precipitating viral proteins and is now used routinely. For analytical purposes, cells are grown in 24 well plates (Falcon), each well having a 2 cm^{2} surface area containing 1-2x10^{5} cells. Following infection and labelling as described above, 100 microliters of lysis buffer is added to each well and the clarified lysate prepared as described above. Fifteen microliters of specific HMAF is then added to the lysate and incubated from 4 hours to overnight at 4^{o}C. Protein A-sepharose is then added, the lysate is incubated for 10 minutes at 4^{o}C, and the agarose beads with the adsorbed immune complexes are pelleted. Following 3 serial washings of this complex in lysis buffer the final pellet is solubilized and prepared for SDS-gel electrophoresis.

Alternatively, we have found recently that these immune complexes can be prepared for isoelectric focusing and 2-dimensional gel analysis by dissolving the precipitate in 2% triton X-100, 8 M urea, and 5% mercaptoethanol. These compounds free the precipitated antigens, are compatible with isoelectric focusing procedures, and must be included in the focusing gels, with the exception of mercaptoethanol which inhibits polymerization.

B. Viruses, cells, media

Baby hamster kidney cells (BHK-clone 15) and cloned virus seeds were obtained from Dr. Joel Dahlrymple, WRAIR. Virus cultures were plaque-purified and stock cultures prepared in either vero or BHK cells. Vero cells were obtained from the American Type Culture Collection. These cell lines were maintained in Eagle's minimal media (MEM) supplemented with 10% fetal calf serum.

C. Polyacrylamide gel electrophoresis

Discontinuous gel electrophoresis was carried out in a 28 cm slab gel apparatus (Bio-Rad Laboratories, Richmond, California) using a discontinuous system modified from Laemmli (12). DATD (N, N'dialytartardiamide) was used as a cross-linker rather than Bis-acrylamide due to its better resolution of glycoproteins. The ratio of acylamide to DATD was 30:1.6, and the stacking gel and the resolving gel were 5% and 13% respectively. The sample buffer contained 2% sodium dodecyl sulfate (SDS), 5% mercaptoethanol, 12% glycerol, 0.005 M phosphate buffer (pH 7.0), and 5-10 µM phenylmethylsulphonyl fluoride (PMSF) as a protease inhibitor. All reagents were purchased from Bio-Rad Laboratories. Samples were dissolved directly in sample buffer (preheated to 100°C) and boiled for 2 minutes. Electrophoresis was carried out using constant voltage (60-90v) for 14-18 hours. The gels were then fixed and stained with coomassie brilliant blue.

D. Fluorography

To allow autoradiographic presentation of tritium-labelled gel profiles, gels were impregnated with PPO according to the procedure of Lasky and Mills (13). Gels were then dried onto filter paper or cellophage and exposed to Kodak BB-5 x-ray film at -70°C.

IV. Identification of Sandfly Fever Virus-specific Proteins

1) Structural Proteins and Glycoproteins

Non-structural proteins have not previously been detected in cells infected with bunyaviruses (3). Therefore, since structural proteins represent the base-line to which our studies of both intracellular virus-specific proteins and protein processing are compared, the identification of all structural proteins is critical. Since most of our experiments have been conducted with Karimibad and Punta Toro viruses, the structural proteins of these viruses have been analyzed in detail. In the first annual report (1979), two procedures were used to identify structural proteins. In the first experiments, media from infected cultures were clarified at 10,000 Xg and virions were pelleted by ultracentrifugation. Virus pellets were then disrupted in lysis buffer and immunoprecipitated as described above. In the second set of experiments, clarified media from infected cultures were concentrated by polyethylene glycol, ultracentrifugation or by amicon ultrafiltration, and subjected to centrifugation in equilibrium density gradients. Fractions containing peak infectivity titers were collected and analyzed directly. In both types of experiments the fluorographs from SDS gels revealed the presence

of polypeptide species thought to be virion structural proteins. However, sandfly fever viruses (2, 8) and apparently bunyaviruses in general (3) are unstable in these gradients and the gradient fractions also showed numerous other polypeptides of presumed cell origin. Also, it could not be verified that all viral structural proteins were detected in immunoprecipitates from solubilized viral pellets since it could not be certain that the HMAF contained activity against all structural proteins.

Therefore, to rigorously identify virus-structural proteins we have concentrated clarified media supernatants from infected cells by ultrafiltration, and precipitated undisrupted virus particles directly by immunoprecipitation. Such precipitates are free of detectable host cell debris by electron microscopy (1979 annual report) and free of labelled host proteins which have been added prior to immunoprecipitation in mixing experiments. These studies should reveal all structural proteins of the virus, irrespective of whether the HMAF contained antibodies to all such proteins, provided (as demonstrated) that activity existed against some surface determinant. The results of such an experiment with Karimibad virus monitored by SDS-gel electrophoresis are presented in Figure 1. Despite the intentional overexposure of the fluorograph, only 4 protein species entered the resolving gel. Their molecular weights are 25,000, 58,000, 60,000 and greater than 140,000 daltons. Results consistent with this analysis have been presented by Robeson et al (1).

The 58,000 dalton and the 60,000 dalton species are rarely resolved by one-dimensional discontinuous electrophoresis. This is due not only to their similar molecular weight, but also to the fact that they are glycosylated (see Figure 4 or 10). We do not feel that these bands represent a single polypeptide species with microheterogeneity in associated oligosaccharides since in the presence of tunicamycin, a potent inhibitor of asparagine-linked glycosylation (see section VI-4), two bands are clearly resolved. Considering the negative-stranded nature of these viruses, the high molecular weight protein is most likely a virion-associated transcriptase. The 25,000 dalton polypeptide has been isolated from equilibrium potassium tartrate gradients at densities in excess of 1.28 g/cc, in association with a tritiated-uridine label, and in the absence of other viral proteins. This protein therefore represents the capsid protein of the viral ribonucleoprotein (RNP). To date, we have not detected the high molecular weight protein in combination with viral RNP. For convenience these proteins will be referred to as VP-25, VGP-58, VGP-60, and L.

Since no other proteins were detected by these procedures we are confident that these four species represent all of the virion structural proteins and consequently, that the HMAF contains antibodies against all structural proteins. Similar studies have been carried out with Punta Toro virus and the results have also confirmed the data, presented previously (1979 annual report), of 4 major polypeptide species: two glycoproteins, VGP-57, and VGP-65, a capsid protein VP-26, and a large molecular weight protein in excess of 140,000 daltons, L. These combinations of structural proteins are consistent with results obtained with other bunyaviruses (3) and with the studies of Robseson, et al. (1). In some preparations of Punta Toro virus, immunoprecipitated polypeptides which migrate slightly slower

and slightly faster than the major capsid band are observed. The significance of these bands is presently unknown, although, as shown below, similar bands are also seen from Sicilian and Itaporanga virus-infected cells.

2) Precipitation of Virus-specific Proteins from Infected Cells.

With the identity of the structural proteins known, indirect precipitation of the Karimibad virus-specific proteins present in infected cells was performed. Vero or BHK cells were infected with Karimibad virus at a multiplicity of 5 and incubated for 15 hours in MEM media containing 2% fetal calf serum. At 16 hours after infection the infected monolayers were washed twice with leucine-free media, and labelled for 5 hours with 50 µCi of ^{3}H-leucine in media otherwise deficient in leucine. Uninfected vero or BHK cells served as controls and were treated identically. Lysates of each culture were then prepared for immunoprecipitation and divided into two equal aliquots, one of which received HMAF specific for Karimibad virus, the other normal mouse ascitic fluid (NMAF). Immune complexes were then allowed to form overnight at 4^{o}C and were subsequently precipitated with solid-phase protein A (Pharmacia). These precipitates were dissolved in electrophoresis sample buffer, boiled, and analyzed on discontinuous gels as described in Section III.

The fluorograph of this gel (Figure 2) shows that each of the structural proteins described in the preceding section can be detected in both infected BHK and vero cells (VP-25, VGP-58-60, and L). In addition to these known structural proteins, a heavily labelled polypeptide with an observed molecular weight of 29,000 daltons is resolved from both cell types and is clearly absent from controls. This protein is of unknown function but would appear to be specified by the viral genome. It does not incorporate 2-^{3}H-mannose and is therefore presumably not a glycoprotein (Figure 4). This polypeptide will be referred to as NVP-29. A polypeptide with an electrophoretic migration identical to Karimibad NVP-29 has been isolated from vero cells infected with Sicilian virus (see below).

Two other polypeptides from Karimibad virus-infected cells, a diffuse band with an electrophoretic mobility corresponding to about 51,000 daltons and a distinct band with an apparent molecular weight of 74,000 daltons, are also uniformly observed in both types of infected cells. However, polypeptides with somewhat similar mobilities (albeit present in much lower amounts) have also been seen sporadically in uninfected cells. Therefore, it is less certain (although it is probable) that these are virus-coded proteins. Additional data to support viral specificity of these proteins comes from two other types of experiments.

In the first, immunoprecipitations of artificially mixed lysates from labelled uninfected cells and unlabelled infected cells do not detect either of these proteins. Consequently, it is unlikely that they represent host proteins which stick to immunoprecipitates nonspecifically. In the second type of experiment we have shown that the synthesis of the 74,000 dalton protein decreases as a function of graded dose of ultraviolet light given to virus preparations before being used for infection (Figure 3). This experiment is a preliminary experiment designed to assess the feasibility of applying UV transcriptional mapping procedures to this group of viruses. The inactivation of viral genes and the consequent effects on viral protein synthesis can be used to determine if monocistronic

or polycistronic messengers are made in this virus system (see renewal proposal, Section III-B). It can be seen that the inhibition of synthesis of individual viral proteins is roughly a function of their molecular weight. However, in this experiment the dose of UV light was too high to accurately plot the inactivation of individual proteins. Additional problems in the interpretation of this experiment are discussed in Section III-B of the renewal proposal. These results are presented here to demonstrate that the 74,000 molecular weight protein is not synthesized if viral genes are inactivated by UV prior to infection, whereas other proteins known to be host proteins are not affected. These data thus suggest that the 74,000 dalton protein is, in fact, virus-specific. In addition, the synthesis of this protein is not detected until 8-10 hours after infection, at times which correspond to the appearance of the virion glycoproteins (see below).

Thus, the only alternative to viral specificity is that this protein is a host protein which is induced as a consequence of virus infection. We have found that in very high concentrations of DRB, the synthesis of this protein is decreased more rapidly than that of the known structural proteins (figure 20). Although other interpretations are possible, this data could be used to argue in favor of host-specificity.

The 51,000 molecular weight protein is glycosylated (figure 4). The appearance of this band is increased in samples of low specific activity and in which the concentration of antibody protein is correspondingly increased. As the heavy chain of IgG molecules is 55,000 daltons, it is our impression that protein overload occurs in this region of the gel with samples of low specific activity resulting in a decrease in resolution of VGP-58-60. It is also possible that this protein is equivalent to the 50,000 dalton protein described by Robeson, et al. (1) from unreduced Karimibad virus preparations. Collectively, the data would suggest that both of these proteins are virus-specific, although in the absence of more definitive proof this cannot be definitely stated. Consequently, they will be referred to as P-51 and P-74 until additional data can be obtained to prove viral-specificity.

We have also initiated immunoprecipitation analysis (using homologous HMAF) of lysates prepared from other sandfly fever virus-infected cells, specifically Naples, Sicilian, Itaporanga, Chagres, and Candiru. The results indicate that an overall similarity in virus-specified proteins will be found throught the phlebotomus fever virus group. However, the polypeptide profiles from each virus also show unique characteristics (figures 5 and 6) which suggest that these techniques could be used for a rapid means of identification.

Irrespective of the virus strain used, the polypeptide species which is synthesized in the greatest amount, and which can be seen in whole cell extracts without immunoprecipitation, is a polypeptide with an electrophoretic migration similar to Karimibad VP-25, and is thus assumed to be the capsid protein. The capsid polypeptides of Chagres, Itaporanaga, and Karimibad show identical migration and thus all have a molecular weight of 25,000 daltons. The capsid proteins of Punta Toro, Candiru, and Sicilian viruses are slightly larger, with an estimated molecular weight of 26,000 to 26,500 daltons. The equivalent protein from Naples virus infected cells migrates to a position intermediate between these two groups (25,500-26,000 daltons).

The immunoprecipitates prepared from each of these virus-infected cultures also show heavily labelled polypeptides with migration similar to the known Karimibad and Punta Toro virus glycoproteins. Similar to the results obtained with Karimibad virus, these polypeptides from Itaporanga, Candiru, Sicilian and Chagres virus-infected cells are usually recovered in a single diffuse band, and only rarely are resolved into multiple distinct species. Among the viruses we have examined, only Punta Toro and Naples consistently give two discrete bands in this molecular weight range.

With regard to the diffuse nature of the glycoprotein bands, these results are undoubtedly related to differential glycosylation of these proteins. Microheterogeneity in glycosylation of Sindbis virus proteins E_1 and E_2 gives rise to a situation in which three and sometimes four bands can be resolved on these DATD-crosslinked gels. In our hands, only after neuraminidase treatment do E_1 and E_2 consistently separate cleanly. Although we will show below that the glycoproteins of Karimibad and Punta Toro contain only polymannose oligosaccharides, and hence contain no sialic acid, the oligosaccharide structure of these other sandfly fever viruses is unknown, and microheterogeneity also exists on polymannose chains. Consequently, until these apparent glycoprotein species are precipitated and analyzed from infected cells treated with tunicamycin, or separated by other procedures such as isoelectric focusing, the number of polypeptides in these bands cannot be determined. However, it is clear that these polypeptide species from different viruses do not all co-migrate. The apparent molecular weights are: Chagres, Sicilian (and Karimibad) 58-60,000 daltons; Candiru 64-66,000 daltons; Itaporanga, 67-69,000 daltons; and Naples, 58-60,000 and 51,000 daltons.

In addition to these presumed structural proteins, lysates from Sicilian virus-infected cells show a heavily labelled band migrating with Karimibad NVP-29. This protein has not been detected in other viruses, although lysates from cells infected with Itaporanga virus show a distinct band above the capsid band and a heavily labelled band at 33,000 daltons, both of which could be non-structural proteins. The 74,000 dalton protein described above is also apparent in cells infected with Naples, Sicilian, and Candiru viruses.

V. Kinetics of Karimibad Virus-specific Protein Synthesis

In order to determine the time of onset and periods of maximal viral protein synthesis following infection, and hence the optimal times for labelling in the experiments reported below, Karimibad virus-infected vero cultures were labelled at sequential 2 hour intervals. Replicate cultures were prepared and infected in 24-well plates (Falcon), labelled with ^{3}H-leucine at the times shown in figure 7, and immunoprecipitated as described in Section III. The results demonstrate that at this multiplicity of infection, viral protein synthesis is first detected at 4-6 hours post-infection, but at this time is limited to VP-25 and NVP-29. Initiation of synthesis of VGP-58-60 and of P-74 is delayed until 8 hours after infection. In other experiments in which higher multiplicities of virus were used for infection, both of these times were reduced by approximately 1 hour, although the relative difference in the synthesis of these two groups of proteins remained constant. After 8-11 hours post-infection, these proteins appear to be made at constant rates.

An assumption inherent in the design of this experiment is that the differences observed are, in fact, related to the time that these proteins are synthesized rather than to different efficiencies of immunoprecipitation. Although it is difficult to control against this latter objection, we believe the former to be the case since antibodies were added in excess, and all immune complexes were precipitated with protein A-sepharose. Since this technique does not require lattice formation between antigens and immunoglobulins, and second immunoprecipitations do not demonstrate residual viral antigens, it is unlikely that proteins are synthesized in appreciable amounts and not detected.

Therefore, there would appear to be temporal controls placed on either viral transcription, viral translation, or both. If this is true, it is tempting to speculate (due to their simultaneous appearance and in approximately equimolar proportions) that both VP-25 and NVP-29 are specified by the same genome segment, and that VGP-58-60 and P-74 may be similarly related. Gentch, et al., have shown through mutant analysis that the small segment of LaCross virus codes for the viral nucleopside protein whereas the viral M segment codes for the two glycoproteins (14, 15). If this is also true for Karimibad virus, it is potable that the molecular weights of the S and M segment, 0.8×10^6 and 2.2×10^6 respectively, could theoretically code for approximately 80,000 and 220,000 daltons of viral protein, respectively, and hence could easily accomodate the relationships proposed above. This could be determined either by in vitro translation of separated virion messengers, by UV transcriptional mapping procedures, or by analysis of the intracellular protein induced by cloned recombinants. These experiments have not yet been performed, although the first two are suggested in the accompanying proposal.

VI. Post-translational Processing of Virus-specific Proteins

The ability to label, immunoprecipitate, and identify virus-specific proteins has enabled us to describe some of the post-translational processing events which occur in cells infected with Karimibad and Punta Toro viruses. Such studies are not only of intrinsic interest with regard to a characterization of the proteins themselves and to their functions in viral replication and morphogenesis, but also may indicate the manner in which the expression of the viral genome is controlled. The last point refers to the fact that there are clearly more virus-specific proteins than genome segments. Consequently, at least some segments must either produce multiple monocistronic or polycistronic messengers since there appears to be no internal initiation on eukaryotic messenger RNA's (see Section III-B, renewal proposal). Polycistronic messengers assume the synthesis of precursor proteins which must be proteolytically cleaved (to form mature proteins) during post-translational processing steps. Therefore, we have attempted to identify precursor proteins in cells infected with sandfly fever viruses. Such studies have revealed several processing steps which apparently take place on viral proteins and in addition have given preliminary suggestive evidence of a polycistronic precursor protein. A processing step of major importance is the glycosylation of proteins destined to be virion membrane proteins. We have characterized the oligosaccharides attached to viral glycoproteins and will describe an oligosaccharide pattern which is apparently unique to these viruses. We have also obtained data which indicates that at least one of the glycoproteins in Karimibad virus is transmembranal during morphogenesis and this data will be described in Section VII.

Search for Precursor Proteins

The standard procedure for the demonstration of polycistronic precursor proteins is to pulse for short periods of time with high levels of radioactive amino acids, and subsequently to demonstrate metabolic instability of high molecular weight precursors with a concomitant increase in mature proteins during a chase period in the absence of label. The sum of the molecular weights of the mature proteins should equal the molecular weight of the putative precursor. Such methods have been successful in revealing picornavirus precursors and their cleavage patterns (16), but have met with less success elsewhere, in systems later shown to contain bonafide precursor polypeptides (17, 18, 20). The chances for success of pulse-chase procedures are a function of the time interval between translation and cleavage. Thus, the relationship between the PE_2 and E_2 Sindbis proteins is easily demonstrable because the cleavage of PE_2 is delayed until final maturation of the virion (19). On the other hand, the cleavage of the 130K Sindbis precursor and the 100 K presumed precursor are co-translational events which are not demonstrable by pulse-chase methodology, and have been shown only by an analysis of temperature-sensitive mutants or in the presence of proteolytic inhibitors (19, 20).

By labeling cells infected with Karimibad virus with 200-300 µCi/ml we have been able to detect all of the viral proteins described above using labeling periods as short as seven minutes. Following this time period, one culture was plunged into an ice bath and prepared for immunoprecipitation at $4^{o}C$ and in the presence of PMSF. An identical culture, similarly labeled, was washed 3 times with medium containing 20 times the normal concentration of "cold" leucine, incubated for an additional hour in this medium, and prepared for immunoprecipitation in a manner identical to the pulse-labeled culture. These experiments have consistently yielded the same results, which specifically are that all known and suspected viral proteins are detected both in the pulse-labeled and chased cultures with no relative difference observed (data not shown). Thus, if proteolytic cleavages are involved in the post-translational processing of Karimibad virus proteins they are not demonstrable by these procedures.

A priori there could be two reasons for this failure to show precursor molecules, assuming that they exist. First (and most likely), cleavages occur so rapidly (i.e., are co-translational) that they are completed within the seven minute pulse and are thus not detected. Second, precursors exist but they are not recognized by the antibody preparation used in these experiments. The latter possibility, however, is deemed unlikely because: 1) it will be shown below that some cleavages (although not necessarily polycistronic) occur, and these precursors have been detected; 2) non-glycosylated Karimibad virus proteins produced in the presence of tunicamycin are efficiently precipitated, indicating that underglycosylation would not effect precipitation of precursors; and 3) precursors have been identified in other viral systems with antibody prepared in a manner similar to that used here or against only structural viral proteins (16, 24, 25, 26).

Therefore, we have undertaken experiments in which Karimibad and Punta Toro virus proteins were labeled in the presence of inhibitors of proteolysis in an attempt to force the accumulation of precursor molecules. Two types of inhibitors

have been employed - both of which have been successful in other systems. In the first, amino acid analogues are used which are incorporated into proteins which then become less suitable substrates for proteases. Alternatively, if the protease activity is newly synthesized, either virus or host-coded, the incorporation of amino acid analogues may inhibit the function of these enzymes directly (16, 17, 24). The analogues, which have been used are 1) ethionine (1.8 millimolar), an analogue of methionine; 2) DL p-fluorophenylalanine (2.5 millimolar), an analogue of pheynlalanine; 3) L-azetidine-2-carbobxylic acid (3.4 millimolar), an analogue of proline; and 4) canavanine (3.3 millimolar), an analogue of arginine. The second means which has been used to inhibit proteolysis is by the use of zinc, an inhibitor which has been shown to be useful in studying cleavage patterns in several systems, notably picornaviruses (16), alphaviruses (21), and several normal cell proteins and glycoproteins (22, 23).

Effect of Amino Acid Analogues

As might be expected, we have found that the incorporation of the amino acid analogues is inefficient if they are added in the presence of their normal counterparts. Consequently, labeling of viral proteins in the presence of these analogues has been done in Earle's balanced salt solution containing the analogues, 2% dialyzed fetal calf serum and tritiated-leucine. (Current experiments are being performed in MEM without arginine, proline, phenylalanine, or methionine, but which is otherwise complete).

In these experiments and the others reported below, infected cells are pretreated with the analogues, and labeled with tritiated-leucine at 16 hours after infection for 4 hours prior to immunoprecipitation. The effect of this mixture of analogues on the gel profiles obtained from Karimibad virus-infected cells and the effect of various times of analogue pretreatment is shown in figure 8. Several alterations are immediately apparent. In the one hour and two hour pretreatment samples, Karimibad VP-25 and NVP-29 are not detected, and only a trace of these proteins is detected following a 1/2 hour pretreatment. Since it was shown above that the initiation of synthesis of these two proteins occurs at the same time, the present observation strengthens the suggestion that their synthesis is, in some way, related. The same argument could be extended to suggest that the synthesis of P-74 and of VGP 58-60 is independent of these two proteins.

The second major alternation apparent from figure 8 is that Karimibad VGP 58-60 is also undetected and is apparently replaced by another species with a slightly slower electrophoretic migration, which is nonetheless produced in large amounts. Therefore, the action of these inhibitors occurs rapidly and is without gradient effect provided that they are added at least one hour prior to labeling. This is presumably necessary for the dilution of existing pools of normal amino acids. The heavily labeled band of 43,000 daltons is also detected in uninfected cells and is most likely actin. However, this band usually is more heavily labeled in immunoprecipitates from infected cells than from uninfected cells. This could be explained either by the fact that a viral-specific protein co-migrates with this band, or alternatively, that a host protein complexes to

the immunoprecipitates (i.e., is "sticky"). Two-dimensional gels are presently being run to resolve this question.

Shown in figure 9 are the results of an experiment in which the effect of each inhibitor was analyzed independently. The results show that individually (Lanes A-1, A-2, A-3, and A-4), the effect of these inhibitors on the synthesis of Karimibad virus proteins is less pronounced than is seen with the complete mixture (Lane A-5). The capsid protein is detected when the inhibitors are added individually through in amounts less than those seen in uninhibited controls (Lane A-6). These results also show that canavanine is clearly responsible for the major effect seen on VGP-58-60. It can also be seen from these data that the observed molecular weight of P-74 increases in the presence of canavanine alone and in the mixture of analogues. A second electrophoretic separation of these samples illustrates the reproducibility of these altered migration patterns (B-1, B-2, B-3, B-4).

To determine if similar effects would be observed with other sandfly viruses, identical experiments have been carried out with Punta Toro virus (C-1, C-2, C-3, C-4). The results show that both viral glycorpoteins of Punta Toro are observed at a higher apparent molecular weight in the presence of canavanine. These data also show that an additional protein of 85,000 molecular weight can be seen and is affected in a manner comparable to P-74 isolated from cells infected with Karimibad virus.

Figure 10 shows the data collected from another experiment in which cells infected with either Punta Toro or Karimibad virus were treated with the complete mixture of analogues and labelled either with ^{3}H-leucine or 2-^{3}H mannose. Although the incorporation of mannose was minimal, the distribution of this label clearly follows the distribution of the leucine label in the presence or absence of analogues. Therefore, these proteins showing decreased migration are, in fact, closely related to the glycoproteins and are not derived from unrelated proteins (for example, a possible precursor to Karimibad VP-25 and NVP-29). This experiment also shows that the synthesis of the Punta Toro virus capsid protein seems relatively less susceptible to the presence of these analogues than the equivalent Karimibad virus protein.

Although canavanine-containing proteins would be expected to be poor substrates for proteases, Aliperti and Schlesinger have shown that Sindbis virus-specific polypeptides containing canavanine can be cleaved, although with a decreased efficiency, at least in their in vitro translation system. These experiments have suggested an auto-protease activity for the Sindbis virus capside proteins (17). Figure 11, however, shows that neither the altered Karimibad glycoproteins or the altered P-74 protein will chase if infected cells are incubated for two hours in the presence of arginine (Eagle's medium).

Discussion

These experiments have revealed that two major analogue-induced changes occur in the observed virus-specific proteins in cells infected with sandfly fever viruses. First, under the appropriate conditions the production of Karimibad VP-25 and NVP-29 proteins is inhibited while the other Karimibad (and Punta Toro) virus proteins continue to be detected. Since amino acid analogues

presumably inhibit only those proteins into which they are incorporated, and the effect is seen within a one-half hour period, it would seem unlikely that the synthesis, per se, of VP-25 and NVP-29 would be prevented. However, uncleaved proteins which could be precursors of these two proteins have not been detected. Therefore, the reasons for the increased sensitivity of these proteins remain unclear. Secondly, under these same conditions, the viral glycoproteins of Karimibad and Punta Toro virus and the P-74 protein from Karimibad virus-infected cells continue to be synthesized in approximately normal amounts, although their migration in gels is altered.

Two questions should now be addressed: 1) what is the nature of the alteration responsible for altered gel mobilities, and 2) what conclusions can be drawn with respect to post-translational modification? With regard to the first question, it is clear that mobilities in SDS gels are usually an accurate reflection of molecular weight, however, this is not always the case. Noel, et al. (27) have described a single amino acid substitution in a histidine-transport protein which drastically alters its migration in SDS gels. However, it is unlikely that the mere substitution of canavanine, per se, in these viral proteins accounts for their altered mobility. This conclusion follows from the fact that several contaminating host proteins and the viral capsid protein which are synthesized in the presence of canavanine alone are not altered in their migration. Since the capsid protein undoubtedly has many basic amino acids to complex with the polyanion RNA it would be expected to contain many arginine residues and, in fact, incroporates more ^{14}C-canavanine (DL-guanido-^{14}C-canavanine hydrochloride, Research Products International, Elk Grove, Illinois) than other viral proteins (data not shown). Therefore, (in reference to the second question), it is highly likely that the decreased migration of these proteins in the presence of canavanine (and in the mixture of amino acid analogues) is due to the fact that they contain additional amino acid sequences, and thus are normally converted to the usual viral proteins by a proteolytic cleavage.

A priori, it could have been expected that canavanine would have had the greatest effect among these analogues in the inhibition of this cleavage, due to the frequency with which arginine residues are found to be the preferred substrates for proteolytic enzymes. The fact that these cleavages are not detected in pulse-chase experiments, in the absence of inhibitors, indicates that proteolytic processing occurs as a co-translational event or very soon thereafter.

These experiments were originally designed to detect translation products of polycistronic messengers and have not as yet done so. However, other cleavage reactions have been detected and are likely related to one of two processes. In the first instance, it is possible that we have prevented the cleavage of "signal peptides". These peptides are sequences on the N-termini of proteins which recognize receptors on endoplasmic reticulum membranes and mediate the complete transfer (secreted proteins) or partial transfer (membrane proteins) of the polypeptide into the lumen (28). The signal sequence is then normally removed, which may have been inhibited in these studies by the incorporation of analogues. This would indicate that the nonglycosylated P-74 as well as the glycoproteins are membrane-associated, a supposition which is, in fact, borne out by our cell fractionation studies (Section VII-3, figure 21). Nonglycosylated proteins

with signal sequences have been isolated; for example, lysozyme (31). However, unlike the glycoproteins, P-74 remains mostly exposed on the cytoplasmic face of internal membranes (see below). Therefore, assuming it is virus-specific, its function could be involved with viral maturation, or conceivably, viral RNA synthesis.

Another possible explanation for these cleavages, at least of the viral glycoproteins, is that they are related to the events which occur in the processing of parainfluenza virus envelope proteins. In this system both envelope proteins are synthesized as precursors which are slightly larger than the mature proteins, generating a situation which is, at least superficially, identical to that described above (32). Although cleavage of these parainfluenza virus proteins is not necessary for virus assembly, particles containing uncleaved glycoproteins show greatly reduced hemagglutinating, neuraminidase, and cell fusion activities, and hence have very low specific infectivities. Activities are, however, regained if particles (or isolated proteins) are treated with the appropriate protease (32, 33, 60).

Hosts vary greatly in the efficiency with which they carry out these cleavages. Thus, Nagai, et al. (60) have shown that multiple cycles in a given host depend on the presence of the appropriate protease as well as the susceptibility of the glycoprotein to cleavage. For Newcastle disease virus (NDV), it was shown that virulent strains were readily activated in all host systems analyzed whereas avirulent strains were activated in only a small fraction (33, 59, 60). Thus, it has been clearly documented in these systems that both host susceptibility and tissue tropisms are a direct function of their relative concentrations of the activating protease of the activating protease. The corollary of this is that mutant viruses with altered glycoproteins can be selected which will grow to high titer in one system and be avirulent for another. Scheid, et al., have isolated a series of Sendai virus mutants which are fully activated by a variety of "non-natural" enzymes, and only these enzymes (59). If the same or similar processes occur in the processing of these sandfly virus proteins, then it must be assumed that vero and BHK cells represent totally permissive cells since uncleaved proteins are not normally found. If, however, the processing of sandfly fever virus glycoproteins is analogous to the situation described for parainfluenza viruses, sandfly fever virus mutants could be isolated with altered amino acid sequences at the appropriate cleavage points as was done for parainfluenza viruses (59). The phenotypic change would be a reflection of a genotypic alteration, and consequently, these mutants would "breed true", unlike those generated by amino acid analogue incorporation. For laboratory production, these viruses would be activated by the "unnatural" protease (e.g., elastase) used for mutant selection (33, 59). Following one round of replication in cell culture, particles would be produced (again showing the altered phenotype) which would have low (but defined) infectivities. Since most antigenic determinants would remain unaltered, these mutant viruses could provide protective immunity (33). In the accompanying proposal we have described experiments which will allow us to translate sandfly fever virus mRNA's in vitro (Section III-B, renewal proposal). Since proteolytic cleavages do not occur in these systems, the glycoproteins should be produced containing extra amino acid sequences, if glycoprotein processing is, in fact, similar to that seen in parainfluenza virus-infected cells. If this is found to be the case the concepts described above will be pursued.

3. Effect of Zinc Ions

As reviewed above, zinc ions have been used in several other systems to prevent proteolysis and hence to detect precursor proteins. Figure 12 represents preliminary data we have obtained which suggests that a high molecular weight polypeptide which is precipitable by Karimibad HMAF can be detected in virus-infected cells. Replicate cultures were infected with Karimibad virus, incubated overnight (16 hours), and then pretreated with leucine-free media containing 0.05, 1.0, or 1.5 millimolar zinc chloride for 30 minutes, and subsequently labelled for 2 hours with tritiated-leucine. One culture from each zinc concentration was then harvested and prepared for immunoprecipitation. Equivalently infected, treated, and labelled cultures were washed and incubated for an additional 2 hours in MEM media devoid of zinc and tritiated-leucine (chase). These cultures were then lysed and immunoprecipitated as above. Mock-infected cultures served as controls.

As can be seen in figure 12, 0.05 mM $zinc^{++}$ was without apparent effect. However, in infected cultures labeled in the presence of 1.0mM zinc, a previously undetected band with an apparent molecular weight of approximately 105,000 daltons can be seen. This polypeptide species was, however, metabolically stable, insofar as it did not "chase". Consequently, its relationship to known viral proteins is unknown. It is also clear that protein synthesis was significantly inhibited in this concentration and was essentially halted in 1.5 mM zinc. We are currently assessing the effects of zinc concentrations intermediate between 0.05 and 1.0 mM. If this finding is reproducible, we will attempt to demonstrate a relationship between this polypeptide and known virus-specific proteins either through pluse-chase methodologies or through a comparison of tryptic peptides. If such a relationship can be detected it would suggest that multiple protease functions are involved in the post-translational processing of these proteins, since the cleavages demonstrated by amino acid analogues were not inhibited in the presence of zinc ions. The observed molecular weight of this protein suggests that it could be a precursor to the envelope glycoproteins as the unglycosylated proteins, produced in the presence of tunicamycin, are approximately 58 and 55,000 daltons. If this is the case, this protein could be equivalent to the B protein seen in Sindbis virus-infected cells. The molecular weight of the protein seen in zinc-inhibited cells also approximate the sum of NVP-29 and P-74. However, if a relationship between these three proteins can be shown, a situation similar to the auto-catalytic cleavage of the Sindbis capsid protein (17) would have to be proposed to account for disproportionate molar ratios of NVP-29 and P-74. Pertinent to this discussion has been the recent finding of Buckmeier and Oldstone that a precursor containing the amino acid sequences of both LCM (arenavirus) glycoproteins can be found in infected cells (26). Since arenaviruses also contain a negative-stranded, segmented genome which produces more viral proteins than the number of segments, it is possible that control of gene expression in arenaviruses and bunyaviruses is similar.

4. Characterization of Sandfly Fever Virus Glycopeptides

a. Introduction

Glycosylation of membrane glycoproteins is a critical step in the replication of most, if not all, enveloped viruses. Inhibition of glycosylation prevents the efficient assembly of alphaviruses (35, 36, 37), rhabdoviruses (38), and orthomyxoviruses(37). Some RNA tumor viruses (37) and herpes viruses (39) produce limited numbers of particles in the presence of glycosylation inhibitors, but these particles have very low specific activities. In addition, the presence of these inhibitors has been shown to prevent cleavage of several viral glycoprotein precursors or intracellular transport of mature proteins (35, 40). Therefore, inhibition of glycosylation represents at least a potential area which may be useful in the design of antiviral drugs (41) and may also be useful in studying the processing of intracellular viral proteins. Furthermore, it has been shown that the solubility of viral envelope antigens is, at least partially, a function of the sugars they contain (40); therefore, this consideration must be taken into account in the design of subunit vaccines.

Also related to this topic are several recent studies which have clearly shown that the rate and site of clearance of either natural or injected proteins is a function of the terminal sugars or the glycosylation patterns which exist on these proteins. Proteins which contain terminal mannose residues are cleared by Kupffer cells or endothelial cells, whereas those containing terminal galactose residues are cleared by hepatocytes (42, 43). It is therefore also likely that clearance and perhaps tissue tropisms of viruses which contain surface glycoproteins may be determined by similar mechanisms. Jahrling, for example, has shown that virulent and less virulent strains of Venezuelan encephalitis virus (VEE) can be separated by differential elution on hydroxylapatite columns, indicating that surface differences exist among these strains (44). He has also shown that after innoculation into hamsters, the less virulent strains are cleared by the liver at the rate of several orders of magnitude per minute,- whereas the virulent strains are not (45, 46). Similar results have been obtained with Western and Eastern encephalitis viruses. Considering that the observed surface properties which differ among these strains must be a function of surface glycoproteins, and that the differential virus clearance bears striking resemblances to the known differential clearance of glycoproteins referred to above, it is possible that strains differing in virulence also differ in glycosylation patterns.

It is normally assumed that glycosylation patterns are a function of the host cell in which the viruses are produced. However, virus-specific differences have been noted in glycosylation patterns among viruses grown in the same host cell (47). Furthermore, glycosylation occurs only on specific amino acid residues in the primary amino acid sequence of proteins (described below) which are specified by viral genetic information. Consequently, one-step virus mutants which differ in these residues could have greatly altered glycosylation patterns which could account for the biological differences noted. Such alterations would be genetically stable.

In addition, if alterations in clearance or virulence could be traced to alterations in glycosylation, lectin chromotography could be used to select for strains with desirable glycosylation patterns, or alternatively, could select against those deemed undesirable. Thus, unlike differences noted among virulent and less virulent strains detected on the basis of oligonucleotide mapping or other purely analytical procedures, alterations detected in glycosylation would have the practical advantage that they could be used to select for mutants with the desired phenotype (and genotype). Related to the last point is the fact that, if the glycosylation patterns of a particular virus or viral glycoprotein are known, lectin columns could be used to purify and concentrate these viruses, or antigens, or remove host contaminants (48). Therefore, the study of the viral protein-linked oligosaccharides is of value from several points of view, and consequently we have characterized the oligosaccharide patterns of selected sandfly fever viruses.

b Background

The carbohydrate units of glycoproteins are covalently attached to the polypeptide chain through either an O-glycosidic linkage between galactosamine and serine or an N-glycosidic linkage between the amide nitrogen of asparagine and N-acetylglucosamine. Although it is clear that O-linked carbohydrate structures are of major importance in several biological systems such as the ABO, MN, and Lewis Blood group antigens (49), O-linked oligosaccharides have not been demonstrated in viruses.

Structural studies on many viral glycoproteins have shown that oligosaccharide units which are linked to polypeptides through asparagine residues have a common inner core structure consisting of two N-acetylglucosamine and three mannose units (50). All of the asparagine-linked carbohydrate chains which have been described can then be divided into those which have only additional mannose residues attached to this core structure (high mannose units) and those termed "complex" units which contain, in addition to the core structure, fucose, galactose, N-acetylglucosamine and sialic acid. The viral glycoproteins which have been analyzed to date have been shown to contain either both complex and high mannose glycopeptides (e.g., Sindbis virus, influenza virus (51, 52) or complex glycopeptides exclusively (e.g., vesicular stomatitis virus,53)).

Studies reported from several laboratories have suggested that N-acetylglucosamine, glucose, and mannose residues are preassembled on dolichol phosphate (an isoprenoid lipid) and this oligosaccharide is transferred en bloc to an asparagine residue on a nascent polypeptide chain (54). Sefton has been able to demonstrate that in Sindbis-infected cells, a preassembled oligosaccharide of approximately 1,800 daltons is transferred from a lipid intermediate to polysome-bound viral polypeptides (51). In similar studies Robbins, et al. have prepared a cell-free extract from cells infected with Sindbis virus which is capable of transferring a lipid-linked oligosaccharide (containing 5 to 6 mannose and 1 or 2 glucose residues) to virus protein acceptors (56).

However, if these oligosaccharide assemblies are the precursors for complex glycopeptides, then clearly considerable processing of these structures must occur to result in the structures found in the mature glycoproteins. Such

processing must include not only the excision of all residues peripheral to the core structure mentioned above, but also the addition of the other monosaccharides found in the complex side chain, and in the proper sequence. The latter processing steps involving the addition of individual monosaccharides is apparently mediated by glycosyl transferases resident in the Golgi apparatus (55). Evidence to support the "trimming" of the transferred intermediates has recently been provided by Tabas, et al. (53) using the vesicular stomatitis virus glycoprotein as a model. However, it is not presently clear whether processing or "incomplete" processing of the same lipid-transferred oligosaccharide also results in the formation of the high mannose side chains.

c. Results and Discussion (figures on following pages)

In order to characterize the glycopeptides of Karimibad and Punta Toro viruses, 2-^{3}H-mannose was used to label cultures which had been infected for 18 hours. To insure efficient incorporation, the labelling media contained one-tenth the normal concentration of glucose. (2-^{3}H-mannose was selected as if it is converted to other sugars, the substituted tritium is removed. Consequently, it can be assumed that the label in glycopeptides is mannose without necessitating identification by acid hydrolysis and paper chromotography.) Following seven hours in this labeling medium, the infected cells were removed in lysis buffer and viral proteins were selected by direct precipitation with homologous HMAF. The specificity of this labeling procedure is indicated in figure 4.

Viral glycopeptides were isolated directly from immunoprecipitates by proteolysis at 37°C with trypsin or pronase (500 µg of enzyme added every 24 hours, for 72 hours, in 10mM Ca^{++}, and 50 mM tris-HCl, pH 8.0). These procedures yield viral oligosaccharides linked to a few amino acids which resist degradation. The size of these mannose-labelled glycopeptides was then determined by Bio-Gel P-6 chromotography and the elution was compared to that of free mannose or to a ^{14}C-labelled oligosaccharide which has previously been demonstrated to contain 8 monosaccharide residues (57). In figures 13 and 14 it can be seen that both Karimibad and Punta Toro virus glycopeptides, prepared with pronase, elute in a sharp peak and with the G_8 marker, although the peaks are broader. Essentially the same results are obtained from typsin-prepared samples, indicating that pronase is more efficient in removing residual amino acids than trypsin. Pronase glycopeptides were used exclusively in the experiments described below.

Unlike the O-glycosidic linkage between galactosamine and serine, the N-glycosidic linkage between N-acetyl glucosamine and asparagine is not hydrolyzed under mildly basic conditions (0.5N NaOH, 20°C, 24 hours). Therefore, this procedure may be used to discriminate between these two possibilities. Following base treatment the electrophoretic mobility of the oligosaccharide is determined at pH 5 by paper electrophoresis. Under these conditions the only charge on the glycopeptide comes from the attached amino acid. Consequently, a serine-linked, mannose-labelled oligosaccharide will remain at the origin due to the base-hydrolysis of its amino acid, whereas an asparagine-linked oligosaccharide will remain positively charged (at this pH) and migrate toward the cathode. Figure 15 shows that following base treatment, all of the Karimibad glycopeptide remains

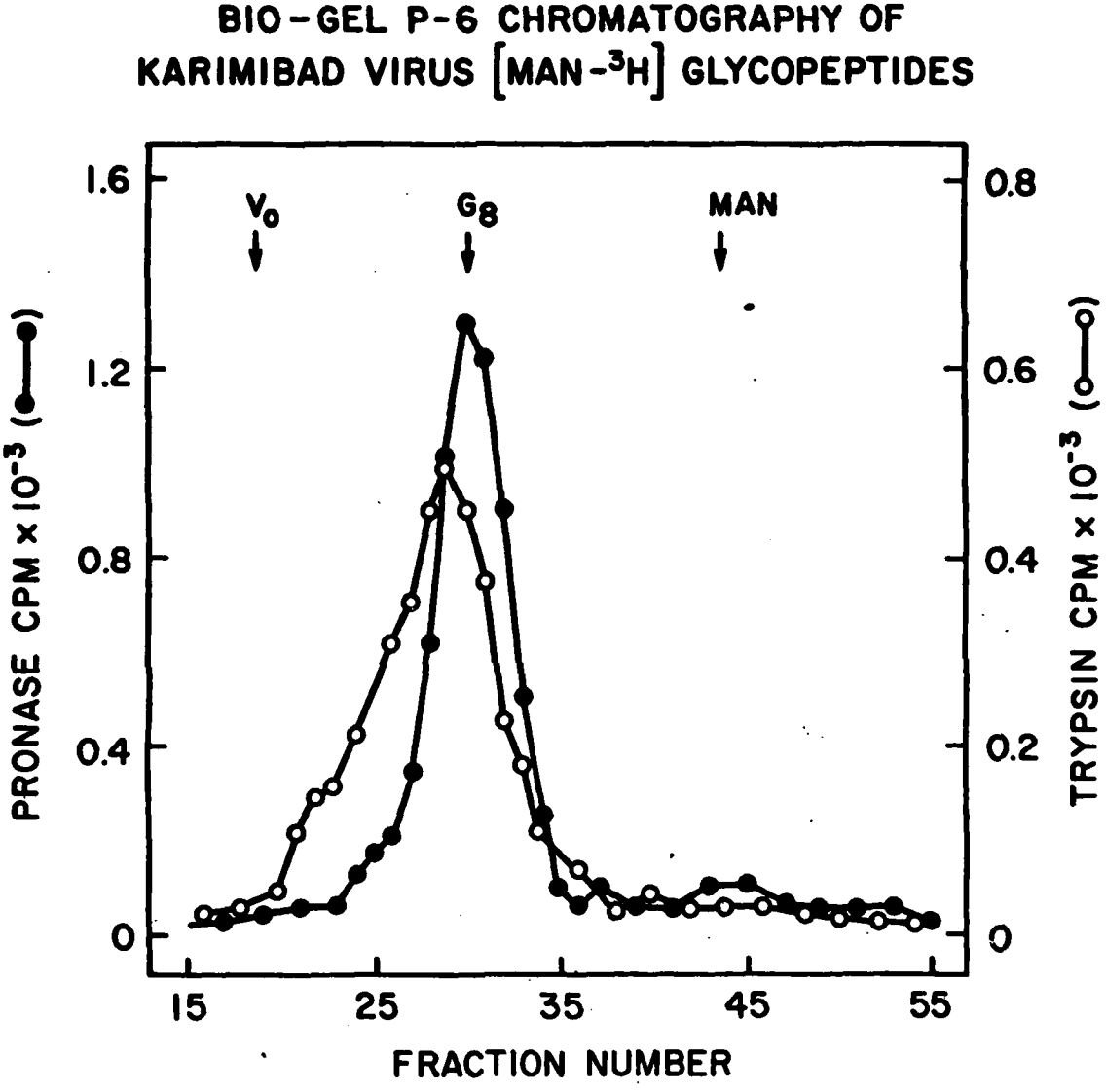

BIO-GEL P-6 CHROMATOGRAPHY OF
KARIMIBAD VIRUS [MAN-^{3}H] GLYCOPEPTIDES
V_o
G_8
MAN
PRONASE CPM x 10^{-3} (●—●)
TRYPSIN CPM x 10^{-3} (○—○)
1.6
1.2
0.8
0.4
0
0.8
0.6
0.4
0.2
0
15
25
35
45
55
FRACTION NUMBER

Figure 13

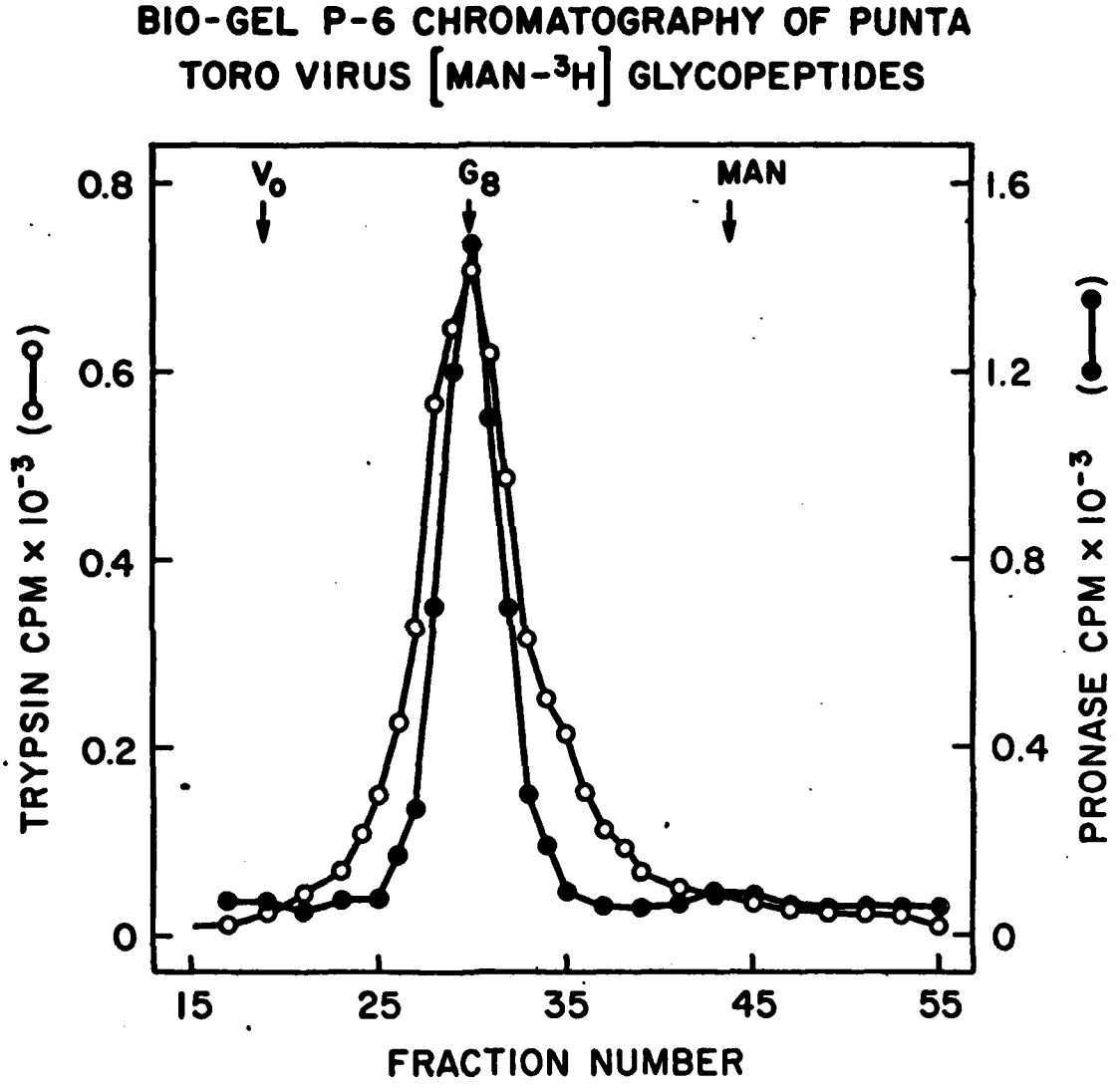

BIO-GEL P-6 CHROMATOGRAPHY OF PUNTA TORO VIRUS [MAN-3H] GLYCOPEPTIDES
Vo
G8
MAN
0.8
0.6
0.4
0.2
0
1.6
1.2
0.8
0.4
0
TRYPSIN CPM x 10-3 (o—o)
PRONASE CPM x 10-3 (•—•)
15
25
35
45
55
FRACTION NUMBER

Figure 14

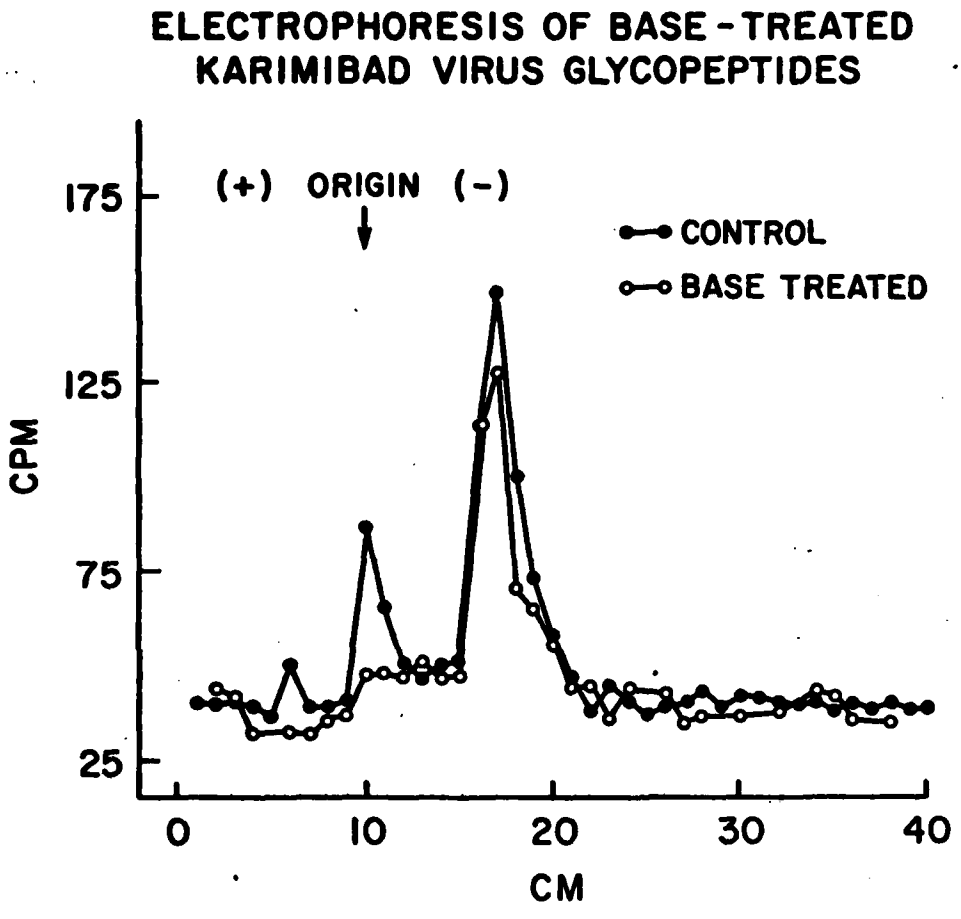

ELECTROPHORESIS OF BASE-TREATED
KARIMIBAD VIRUS GLYCOPEPTIDES
(+) ORIGIN (-)
CONTROL
BASE TREATED
CPM
175
125
75
25
0
10
20
30
40
CM

Figure 15

charged and migrates away from the origin. Therefore, glycosylation of Karimibad virus glycoproteins occurs exclusively at asparagine residues.

This has been independently confirmed by monitoring the effect of tunicamysin on the incorporation of 2-^{3}H-mannose into Karimibad virus glycoproteins, as monitored by immunoprecipitation. Tunicamycin inhibits the glycosylation of the lipid intermediate responsible for the transfer of the preformed oligosaccharide to asparagine residues but it does not affect glycosylation at serine residues (36). Figure 16 shows that in vero cells the incorporation of labelled mannose into Karimibad virus glycoproteins in the presence of tunicamycin is inhibited by 93%. This effect is not seen in infected BHK cells, presumably due to the poor incorporation of the labelled precursor.

As described above, asparagine-linked oligosaccharides occur in two forms: 1) a polymannose type containing only N-acetylglucosamine and mannose, and 2) a complex type in which core mannose groups are linked to peripheral N-acetylglucosamine, galactosamine, sialic acid, and fucose. Consequently, in the complex type of oligosaccharide the mannose residues are not accessible to the exoglycosidase, α-mannosidase, Figure 17 shows the results of an experiment in which Karimibad virus glycopeptides were treated with α-mannosidase and subsequently analyzed by Bio-Gel P-6 chromotography. The results clearly show that following α-mannosidase treatment, most of the mannose label separates from the native glycopeptide and elutes at the position of free mannose (compare with figure 13). The small amount of mannose label which is not removed is presumably that bonded to N-acetylglucosamine which is β-linked and hence resistant to this enzyme.

The conclusions from these studies are:

1. That Karimibad virus glycoproteins are glycosylated exclusively at asparagine residues;

2. That only polymannose type oligosaccharides are present. As complex types are absent, only mannose and N-acetylglucosamine residues are used to construct these oligosaccharides;

3. That the size of this oligosaccharide is sufficient to contain 5 to 6 mannose residues linked to the two N-acetylglucosamine residues;

4. That the size of the Punta Toro glycopeptides is identical to that of Karimibad virus, suggesting that all sandfly agents may be similar;

5. That as no separation of the two glycoproteins was made, and only one type of glycopeptide was found, the two envelope proteins of these viruses are glycosylated in the same manner.

As indicated above, several other enveloped viruses have been examined by techniques similar or identical to those used here, including alphaviruses, rhabdoviruses, influenza viruses and RNA tumor viruses. Among these viruses there is no precedent for the patterns of glycosylation demonstrated to occur in sandfly fever viruses. Although all viral oligosaccharides examined to date

EFFECT OF TUNICAMYCIN (TN) ON THE SYNTHESIS OF KARIMIBAD VIRUS PROTEINS

(IMMUNOPRECIPITATION)

	^{3}H-LEUCINE (cpm)		^{3}H-MANNOSE (cpm)		^{3}H-MANNOSE (cpm) (TN Pretreatment only)	
	+TN	-TN	+TN	-TN	+TN	-TN
VERO	17,829	23,870	2,828	44,333	7,038	43,524
BHK	36,898	32,598	2,373	2,102	2,324	2,532

Figure 16

BIO-GEL P-6 CHROMATOGRAPHY OF α-MANNOSIDASE TREATED KARIMIBAD VIRUS GLYCOPEPTIDES

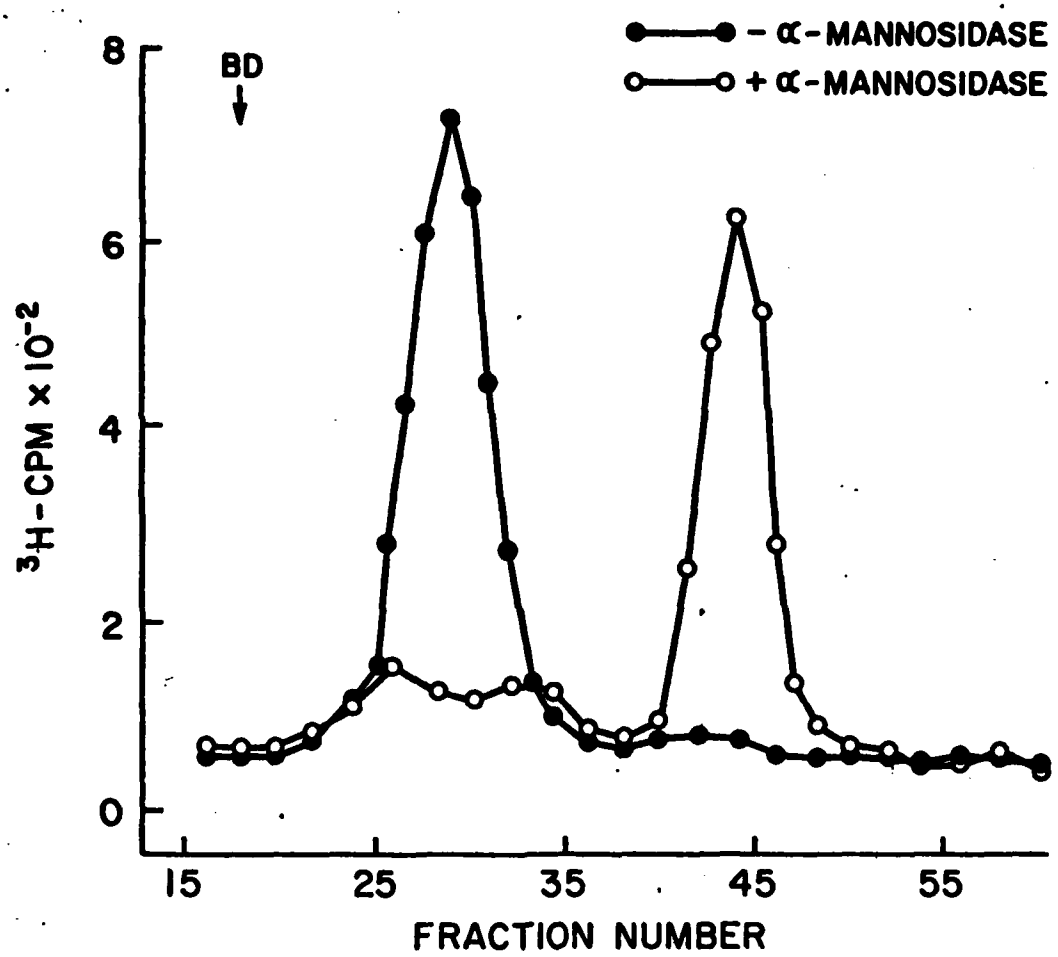


Figure 17

have been shown to be linked to envelope proteins via asparagine residues, all of the other viruses examined contain either both polymannose and complex glycopeptides or complex types exclusively.

It is not immediately clear why sandfly fever viruses should give so different a profile. However, since all of the other viruses which have been studied mature at the plasma membrane, one possibility is that the differences noted relate to the internal maturation of sandfly fever viruses. Although the polymannose core is transferred to proteins immediately following their insertion into the lumen of the endoplasmic reticulum (51, 54), the glycosyltransferases which produce the complex type of glycopeptides are believed to be all located in the membrane of the Golgi apparatus (55). Since sandfly fever agents mature at this membrane it is possible that alterations due to the insertion of viral membrane proteins or other events which occur during budding disrupt these glycosylation mechanisms.

It is clear, however,that the lack of complex type oligosaccharides and hence the lack of sialic acid, galactose, and fucose could be very useful in the purification of these viruses or of their envelope antigens. Since most glycoproteins and membranes contain all of these sugars they could be removed from sandfly fever virus or antigen preparations by reaction with the appropriate lectin, for example, soybean agglutinin (galactose), ulex lectins (fucose), or limulin (sialic acid). Sandfly viruses or their envelope antigens could then be purified from non-glycoproteins by adsorption to, and elution from, Concanavalin A.

VII. Intracellular Distribtuion of Viral Antigens in Infected Cells

In the preceding annual report we presented our initial studies on the morphogenesis of sandfly viruses as monitored by thin-section electron microscopy and on the intracellular localization of viral antigens as monitored by indirect immunofluorescence. Several unresolved aspects of viral replication were revealed by this work, and it was proposed to study these points in more detail. Some of these studies have now been completed and preliminary results will be presented on the remainder. Specifically, the role of nuclei in the replication of sandfly fever viruses, the possible presence of viral antigens on the cell surface (plasma membrane), and the possible transmembranal orientation of the virion glycoproteins during morphogenesis have been examined.

1) Are virus-specific antigens present on the surface of infected cells?

With enveloped viruses which mature by budding at the plasma membrane, envelope glycoproteins are inserted into the plasma membrane and are exposed at the cell surface (19). Such antigens are believed to play a significant role in disease pathogenesis insofar as they represent target antigens which may be recognized by immune surveillance mechanisms. Consequently, infected cells can be killed by specifically sensitized T cells or by complement-dependent, antibody-mediated lysis. Similarly, such virus-coded surface antigens are necessary if cytotoxicity assays are to be used experimentally to monitor T-cell activity. As shown in the previous report and by others (5, 34), the maturation of bunyaviruses occurs internally on smooth membrane vesicles, and only rarely,

if at all, at the plasma membrane. However, release of the virus from infected cells involves the fusion of these vesicular membranes with the plasma membrane. The possibility existed, therefore, that virion glycoproteins, integrated into vesicular membranes which did not become incorporated into maturing virions, would accumulate in the plasma membrane following fusion and exocytosis. This possibility has been studied by immunofluroescence and immune-electron microscopy.

If infected cells are prepared for immunofluorescence by fixation with paraformaldehyde rather than acetone, the cellular membranes are not delipidized and hence immunoglobulin molecules are unable to enter cells - thus restricting their reaction to cell surface antigens (40). Vero cells growing in microslide cultures (Lab-Tek) were infected with Karimibad virus or Punta Toro virus at a M.O.I. of five, incubated for various lengths of time from 8 to 24 hours, and fixed in either acetone or freshly-prepared 2% paraformaldehyd. HMAF specific for these viruses and goat anti-mouse gamma globulin (fluorescein labelled, Cappell Laboratories) were then used in an indirect assay as described previously (1979 annual report).

Acetone-fixed cultures gave results identical to those reported previously. Paraformaldehyde-fixed cultures showed significant membrane fluorescence in infected cells, which was of greater intensity than that seen in uninfected cells (data not presented). However, our experience from thin-section electron microscopy has indicated that significant numbers of released virions are associated with cell surfaces and consequently immunofluorescence would not distinguish between surface-associated virus and viral antigens integrated into the plasma membrane. A similar problem in interpretation would have resulted from studies of surface-specific radioisotope incorporation (lactoperoxidase iodination or galactose oxidase-tritium borohydride procedures - see 1979 proposal). Due to these problems and the fact that our studies of virion glycopeptides (see above) have indicated that viral glycoproteins would not be a suitable substrate for the galactose oxidase procedure, the experiments have not been performed. Rather, this question was addressed by immune-electron microscopy in which ferritin-antibody labelling of surface antigens and surface-associated virus could be distinguished.

Vero cells were infected with Punta Toro virus as described above and fixed with 1% glutaraldehyde. A 1:20 dilution of Punta Toro virus-specific HMAF was reacted with these fixed cells for 30 min at 4°C and then washed exhaustively with PBS, followed by treatment with ferritin-conjugated sheep anti-mouse gamma globulin for 30 min at 4°C. Cells were then washed again and prepared for electron microscopy as described previously (19, 1979 annual report). These studies have revealed a very heavy ferritin labelling of surface-associated virus and a very sparse ferritin labelling of cell plasma membranes (figures 18a, 18b) even in cells containing virions and at times after infection when immunofluorescence (acetone-fixed) indicates that all cells are infected. These results are to be contrasted with similar studies we have undertaken with the surface-maturing Sindbis virus (19) which show a very heavy ferritin binding to infected cell plasma membranes even in areas where budding virus could not be seen.

Our experiments clearly indicate that the amount of Punta Toro viral protein integrated into plasma membrane is minimal. However, we have screened approximately 40 sections of both infected and uninfected cells and have gained the impression that the sparse labelling of infected cell membranes is greater than seen in controls. Relevant to this point is an additional experiment which was performed concurrently with the ferritin labelling studies.

In viruses which mature at the plasma membrane, the incorporation of specific antibody into the growth media prevents release of budding virus (61). We have carried out similar experiments to determine if antibody in the media would prevent exocytosis of sandfly fever viruses. For these studies, cells were infected with Punta Toro virus (as above), and grown in standard media to which a 1:20 dilution of heat-inactivated Punta Toro virus-specific HMAF was added at 6 hours after infection (prior to virus release). Controls received normal mouse ascitic fluid. Cells were harvested at 18 hours after infection and processed for thin section microscopy as above.

The results which are indicated in figure 19 show 1) that exocytosis of virus continues in the presence of specific antibody, 2) that surface-associated virus particles are oriented in extended rows on the plasma membrane, and 3) smaller electron dense structures (200 Å in diameter) are linked to virus particles, apparently by the action of antibody since they have never been observed in the absence of antibody. These results suggest several conclusions. As plaque assays would not give useful data, we have no quantitative information on the degree to which antibody may inhibit virus release. However, no difference in the number of virus particles within cells was observed in the presence of antibody, and substantially more were seen outside- presumably due to antibody-induced precipitation. The fact that these antibody-linked virus particles are linearly associated with the plasma membrane suggests that they may be cross-linked to viral antigens present in the plasma membrane. However, we cannot exclude the possibility that vero cells may contain F_c receptors or that virus particles are non-specifically precipitated on to cell surfaces.

Finally, the nature of the structures which are linked to the surface of virions is unknown, although since the combining sites of individual immunoglobulin molecules are identical, these structures must contain viral surface proteins, and hence are unlikely to be viral RNP structures which seem to be present in media concentrates in large numbers (1979 annual report, 8). Superficially, these structures resemble ribosomes, and hence, could be ribosomes containing nascent viral surface proteins released from lysed cells. An alternative possibility is that they may be analogous to the slowly sedimenting viral hemagglutinin which as has been found in flavivirus-infected cells and tissues (62, 63, 64). If this latter possibility is correct, such structures could serve as a source of viral antigen already in micellar organization (see 1979 annual report) which could represent a potent immunogen free of the risks associated with preparation containing viral nucleic acids, and hence should be investigated. In conclusion, these studies have shown that antibody does not prevent the release of sandfly fever virus from infected cells and although we cannot eliminate the possible presence of some viral antigen in the plasma membrane, such an incorporation must occur at a very low efficiency, if at all. The nature of the smaller particles is presently being studied (see below).

2) What is the role of the nucleus in the replication of sandfly fever virus?

On the basis of observations made following indirect immunofluoresence analysis of Punta Toro and Karimibad virus-infected cells, we reported (1979 annual report) that viral antigen was first detectable in a pronounced perinuclear location and later throughout the cytoplasm. However, it was also possible to demonstrate at late times after infection (20 hrs) intranuclear inclusions which apparently contained virus antigen. This is a reproducible observation. Although the percentage of cells showing distinct inclusions (see 1979 report) is low, (1-4%), few nuclei from infected cells are devoid of all fluorescence as is seen in uninfected cells. The question remained as to whether this observation was artifactual or whether the presence of viral antigen in nuclei indicated a role for this organelle in the replication of these viruses.

The former possibility could be explained by cytoplasmic inclusions which underlay or overlay nuclei or by structurally compromised nuclear membranes which could allow viral proteins normally found in the cytoplasm to accumulate in the nucleus. The latter possibility is well documented in influenza viruses in which cellular DNA transcription is required for virus replication (65). A third possibility for the presence of intranuclear viral antigen exists as demonstrated by certain parainfluenza viruses in which viral proteins and nucleocapsid structures do appear in the nucleus, but apparently not as an essential phase of the multiplication cycle (66).

We have extended our original observations for three reasons. First,we were intrigued by the possibility that bunyaviruses which have a segmented, negative-stranded genome like influenza viruses may have similar requirements for viral RNA transcription. Second, we have found that the proteins identified by immunoprecipitation do not always appear in the same relative concentration, possibly because one or more are present within nuclei and the efficiency of their recovery is a function of the degree to which nuclei were disrupted in the preparation of the lysate. Third, we have used canavanine in studies of viral protein processing (Section V-2). This amino acid analogue has been shown by Maeno et al. (67) to inhibit the transport of influenza virus proteins, NP and NS, from the nucleus back to the cytoplasm. Thus, the use of this inhibitor could potentially worsen the situation referred to above, should it exist, and perhaps explain why VP-25 and NVP-29 are not found after treatment with mixtures of amino acid analogues (figure 8).

We have examined these possibilities by characterizing 1) the distribution of viral antigens in infected cells incubated in the presence or absence of amino acid analogues as monitored by immunofluoresence, 2) the viral proteins present in nuclear vs. cytoplasmic fractions of infected cells incubated in the presence or absence of amino acid analogues, and 3) the viral proteins synthesized in the presence or absence of DRB, a specific inhibitor of RNA polymerase II.

1. The effect of amino acid analogues on the distribtuion of viral antigens was monitored in both Punta Toro and Karimibad virus-infected cells. Immunofluorescence following acetone fixation was performed as described previously (1979 annual report). Amino acid analogues (canavanine, ethionine, fluorophenylalanine, and azetidine-2-carobxylic acid) or canavanine alone were used in the same concentrations as in the experiments described in Section VI-2, or in separate experiments, in one-tenth the normal concentration. Cell cultures were infected at a M.O.I. of 5 and incubated in Eagle's media containing 2% fetal calf serum. The analogues were added at either 6 or 9 hours after infection in Earle's balanced salt solution (EBSS) and all cultures were acetone-fixed at 15 hours after infection. The results of these experiments showed that in none of the above combinations did amino acid analogues induce an accumulation of viral antigen in nuclei over that seen in untreated controls, in contrast to the effect of these inhibitors in influenza virus-infected cells (data not shown).

2. The results of the experiments in which viral proteins were immunoprecipitated from nuclear and cytoplasmic fractions in the presence or absence of amino acid analogues were consistent with those of the immunofluorescence experiment described above. Infected cells were treated with amino acid analogues in the concentrations described above from 15 to 17 hours after infection and subsequently labelled with 50 µCi of ^{3}H-leucine for 4 hours. Cells similarly infected and labelled but not treated with analogues served as controls. All cultures were then washed and the cells were removed and lysed in the standard immunoprecipitation buffer containing 1% triton X-100 but lacking deoxycholate. Nuclei were separated from the cytoplasmic lysate (as monitored by phase microscopy) by centrifugation at 1000 Xg. The nuclear pellet was then lysed by the addition of sodium deoxycholate to a final concentration of 0.5% followed by vigorous homogenization in a stainless steel homogenizer with a tolerance of 0.0005" (Kontes, Vineland, New Jersey).

Immunoprecipitation was then carried out utilizing either Karimibad or Punta Toro specific HMAF and solid-phase protein A (Pharmacia) according to our standard procedures. The results showed that the only viral protein present in the nuclear extract was the viral nucleocapsid protein and in concentrations much less than that present in the cytoplasmic fractions. The presence of amino acid analogues did not alter this distribution (data not shown).

3. To determine whether host DNA transcription was necessary for sandfly fever virus replication we have assessed the effect of DRB, a specific inhibitor of RNA polymerase II (the enzyme responsible for the synthesis of eukaryotic messenger RNA), on the synthesis of Karimibad virus-specific proteins. The effect of this inhibitor is equivalent to α-amanitin although unlike α-amanitin it is readily transported into cells (58). Actinomycin D, α-amanitin, or DRB will inhibit orthomyxovirus replication and protein synthesis provided they are added prior to or soon after infection (65). In our experiments, vero cells in a 24 well plastic plate (Falcon) were infected with Karimibad virus (MOI-5) and incubated for 18 hours in the presence of either 0, 10, 100, or 200 micromolar DRB, added at -2, 3, 9, or 18 hours after infection. Medium containing tritiated-leucine and the appropriate concentration of DRB was then added to each well, and the plate was incubated for an additional 4 hours, at which time immunoprecipitates were prepared according to our standard procedures. The results are presented in figure 20.

Two hundred μM DRB is approximately three times that concentration required to inhibit 95% of the activity of RNA polymerase II (58). The results demonstrate that irrespective of the concentration of DRB or the time of its addition, viral proteins continue to be synthesized. There is, however, some inhibition of viral protein synthesis at higher drug concentrations or at earlier times of addition. Nonetheless, it seems clear that Karimibad virus protein synthesis and hence viral RNA transcription does not require host DNA transcription, at least as mediated by RNA polymerase II.

In conclusion, these studies have shown that the positive fluorescence which is seen in the nuclei of some cells infected with Karimibad or Punta Toro virus is most likely due to the presence of small amounts of viral capsid protein, a situation not dissimilar to that seen in cells infected with other negative-stranded viruses (66). It cannot be concluded from these experiments that the nuclei are not involved in the replication of sandfly fever viruses, and it should be noted that Bunyamwera virus does not replicate in enucleated cells (66). However, it can be concluded that the situation seen in influenza virus-infected cells, in which host DNA transcription is required for viral protein synthesis, and in which amino acid analogues cause an accumulation of viral proteins in nuclei, does not extend to sandfly fever virus-infected cells. Results consistent with these students on the effect of DNA-specific transcription inhibitors have recently been published by Vezza et al. (10). Consequently, the removal of nuclei from lysates prior to the immunoprecipitation of viral proteins does not prejudice the results obtained in studies of viral protein synthesis or processing - at least with respect to the presently recognized viral proteins.

3. Distribution of virus proteins with respect to cytoplasmic membranes and role in virion maturation.

As indicated in the first annual report (1979), the final maturation (budding) of sandfly fever viruses occurs on smooth vesicular membranes of the endoplasmic reticulum or the Golgi apparatus. Particulate nucleocapsids (viral RNP) are not seen in the cytoplasm of infected cells prior to their condensation at the time of virion maturation - a finding characteristic of viruses with helically arranged ribonucleoprotein. Following the attachement of viral RNP to the cytoplasmic face of these membranes, virions can easily be observed in all stages of maturation. In budding figures which were sectioned such that the bilayer nature of the membrane was evident, an increased electron density is apparent on the cytoplasmic face, which eventually forms the viral nucleocapsid, and also on the lumen side, which is due to the concentration of viral glycoproteins which eventually form the spikes of the completed virion (1979 annural report and figure 22).

Of interest is the fact that neither of these modifications are seen without the other being present on the contralateral side of the membrane. This suggests that viral RNP can only attach to membranes which have previously been modified with viral glycoproteins and, in addition, presupposes some form of transmembranal recognition, most likely mediated by a transmembranal viral glycoprotein. Similar recognition signals are believed to occur between viral

glycoproteins and the nucleocapsids of Sindbis virus (11, 19), and between the viral glycoprotein and the M proteins of several negative-stranded, helical RNA viruses (68), all of which bud from the plasma membrane. The absence of an "M" type protein in the Bunyaviridae suggests a direct link, at least during maturation, between viral RNP and spike glycoproteins - similar to that between glycophorin and spectrin in the erythrocyte membrane (69).

These morphological observations suggest that following the synthesis of the viral glycoproteins and the insertion of the amino termini through the endoplasmic reticulum membrane (presumably mediated by a signal sequence, 28) the carboxyl terminus of at least one protein remains exposed on the cytoplasmic face and has some affinity for the viral capsid proteins. This can be tested directly by treating membrane vesicles with proteolytic enzymes or crosslinking agents and monitoring the results by electrophoresis following immunoprecipitation. We will present preliminary results we have obtained with the first of these procedures.

These studies require the separation of membrane and soluble fractions from the cytoplasm of infected cells and hence also indicate which of the viral proteins are membrane-bound. When cells are homogenized in buffers of low ionic strength, internal membranes are converted to vesicles which normally are orientated with the cytoplasmic side outermost; such vesicles are closed and are impermeable to protease enzymes (11, 68). Consequently, it is possible to isolate these vesicles and determine which proteins are exposed at the surface by treatment with proteases and monitoring the results by gel electrophoresis. Such an analysis has been carried out with cells infected with Karimibad virus. Since the plasma membrane is not believed to contain significant amounts of viral protein (see discussion above), no attempt has been made to purify various cell membrane fractions. Also, we have not yet proven that the vesicles we have analyzed are, in fact, organized with the cytoplasmic face outermost, although our results are consistent with this interpretation. Future experiments will study only those vesicles which are excluded by lectin-agarose columns which will remove reversed vesicles since glycopeptides are exposed only at the lumen face (70).

Cells infected with Karimibad virus or uninfected cells were incubated for 16 hours in Eagle's media (MEM) containing 2% fetal calf serum. Both infected and uninfected cultures were then labelled for 2 hours in leucine-free media to which had been added 2% dialyzed fetal calf serum and 100 µCi/ml of ^{3}H-leucine. After the labelling period the cells were scraped from the growth surface and 10% of each culture was prepared directly for immunoprecipitation according to standard procedures. The remainder of the cells were suspended in 5 mls of SS buffer (0.01 M Hepes, pH 7.0, 0.01 M KCl, and 0.015 M magnesium acetate), allowed to stand for 10 minutes on ice and subsequently homogenized with 40 strokes of a dounce homogenizer. The homogenates were then pelleted at 40,000 RPM in a Beckman 50.1 rotor for 2 hours. The membrane pellets were resuspended in 150 microliters of SS buffer, divided into 3 aliquots, and treated as follows:

A) received 5 microliters of 0.1 M PMSF only
B) received 6 units of chymotrypsin (Calbiochem)
C) received triton X-100 and DOC, to a final concentration of 1% and 0.5% respectively, and 6 units of chymotrypsin

These treated samples were then incubated for 1 hour in a water bath (37°C), after which time all treated samples received PMSF to inhibit further chymotrypsin digestion. Supernatant fractions over the original 40,000 RPM membrane

pellet were concentrated to 0.5 mls by ultrafiltration with Millipore immersible concentrators and divided into two aliquots. The first received PMSF directly and the second was treated with chymotrypsin followed by PMSF as above. 10X immunoprecipitation buffer was then added to each sample to achieve concentrations normally used for immunoprecipitation, and the resulting immunoprecipitates prepared for SDS-gel electrophoresis. The results are presented in figure 21.

As can be seen in this figure, the capsid protein (VP-25), and glycoproteins (VGP-58-60) of Karimbad virus as well as P-74 were pelleted with the membrane vesicles (Lane A). The 29,000 dalton protein was obtained in supernatant fractions only and in relatively small amounts - suggesting that this protein is not membrane-bound (Lane D). We suspect, although have not yet proven, that the remainder of this protein is bound to the concentrating membrane. From vesicles treated with chymotrypsin (Lane B), it can be observed that 1) the 74,000 molecular weight protein is removed, and 2) a unique 50,000 molecular weight polypeptide species, which is precipitated with specific antibody, appears, and migrates in front of the major glycoprotein band. Since the only protein species of higher molecular weight which contains sufficient radioactivity to account for the density of this new band is the viral glycoprotein species, this protease-induced band must have originated from one or both of the viral glycoproteins by the removal of a specific amino acid sequence. Much of the capsid protein and the glycoproteins in this sample are resistant to the action of this enzyme, presumably due to their presence in mature virions which had budded into vesicles prior to cell homogenization.

Although free virus particles would have been pelleted along with membrane vesicles, it is unlikely that the 50,000 dalton polypeptide arose from protease action directly on virus particles. The evidence for this is that 1) by thin section electron microscopy all intracellular virus particles reside within membrane vesicles, and 2) when vesicular membranes are disrupted by detergents and subsequently treated with chymotrypsin, all of the normal glycoprotein band is degraded (Lane C). However, rigorous proof that free virions are not confusing this analysis will be provided by a similar analysis of vesicles excluded by Concanavalin A columns, which from our studies of virion glycopeptides, we now know will bind virions very efficiently.

Lane C therefore represents the control experiment in which we show that the glycoproteins would have been completely degraded were they not partially protected by membranes (Lane B). Detergent-lysed and protease-treated fractions (Lane C) nonetheless contain substantial amounts of undegraded capsid protein. Whether this results from a native resistance of the capsid protein or of the nucleocapsid itself is unknown. That not all of the 50,000 dalton protein is degraded in this sample suggests that this protein assumes an orientation in protein-detergent micelles similar to its orientation in vesicular membranes and is hence partially protected.

Although preliminary, these data are strong evidence for the suggestions made from thin section analysis of budding particles, that at least one (and perhaps both) of the virion glycoproteins assumes a transmembranal orientation in smooth membranes. From the data presented above, it would appear that approximately

15 percent of its length is exposed on the cytoplasmic face. This "stub", presumably the carboxyl terminus of the protein (28, 68), is then accessible to both chymotrypsin after cell homogenization and to viral capsids during virion morphogenesis. Similar protease-resistant fractions of membrane-associated viral glycoproteins have been demonstrated in Sindbis (9) and VSV infected-cells (68). The complete removal of the 74,000 dalton protein by the protease indicates that this protein is either mostly exposed on the cytoplasmic face of membranes, or alternatively, that its protease-resistant fraction comigrates with other viral proteins.

It is our intention to expand this experimental system to allow a biochemical description of the events which occur in the assembly and maturation of sandfly fever viruses, and this is presented in the accompanying proposal. Our current understanding of the process is discussed below.

4. Discussion

The combination of biochemical, immunofluorescent, and electron micographic data reported here and in the previous report is beginning to allow us to define the events which occur in the morphogenesis of sandfly fever viruses. These concepts will now be briefly discussed. Since Murphy, et al. (5) have shown by thin-section electron microscopy procedures that the morphogenesis of many bunyaviruses appears similar, these general considerations which apply specifically to the sandfly agents described here may extend to other members of the family, Bunyaviridae.

The synthesis of Karimibad virus proteins commences within a few hours after infection and these proteins accumulate in perinuclear areas. This synthesis is initially limited to VP-25 and NVP-29, but by 8-10 hours after infection, all proteins are produced at constant rates. The glycoproteins and P-74 are synthesized in a form which is slightly larger than the mature proteins but these are processed rapidly and all become membrane-bound. Whether larger polycistronic precursor molecules are synthesized or not has not as yet been determined. The glycoproteins are presumably synthesized on membrane-bound ribosomes and are transported at least partially into the endoplasmic reticulum where they receive a polymannose, core-type oligosaccharide. One end of at least one glycoprotein (presumably the carboxyl terminus) remains exposed on the cytoplasmic face. P-74 is probably also synthesized on membrane-bound polysomes but becomes orientated on the membrane in a more peripheral position since it is not protected from proteases and does not become glycosylated. The glycoproteins are then transported to smooth membranes and although some are clearly the Golgi lamellae, further modification of glycosylation does not occur since they contain no complex oligosaccharides. The relative membrane position of P-74 and its function, assuming it is a viral-coded protein, is not known except that it does not become incorporated into mature virus particles.

The capsid protein, either free or complexed with viral RNA, also becomes membrane-associated. Electron microscopy, however, indicates that the binding of viral ribonucleoprotein occurs only at areas of the membrane which contain viral glycoproteins. Since these viruses lack an "M" protein it is highly likely that the ribonucleoprotein has an affinity for the (carboxyl?) end of at least one viral glycoprotein, which, in fact, can be demonstrated to be

exposed on the cytoplasmic face of internal membranes by protease digestion. This interaction of the viral RNP and the viral glycoproteins initiates the budding process. This process includes the continued concentration of viral RNP on the cytoplasmic face, and the continued accumulation of virion glycoproteins into contralateral spike structures. Since membrane proteins are free to move in the plane of the membrane, it is likely that the transmembranal association of the viral RNP with the glycoprotein "caps" these membrane proteins and holds them in place. The association of one entire genome in this manner completes the budding process, the membranes at the base of the "bud" fuse, and a morphologically complete virion is formed. (These processes are diagramatically illustrated in figure 22). How assembly of virions in this manner assures that the correct assortment of genome segments become incorporated into each virus particle, if in fact this occurs, is unknown. Conceivably, there could be some non-covalent bond between the three segments. A similar problem exists for influenza viruses in which the number of segments is greater.

Two considerations would suggest that at least one of the virus glycoproteins remains transmembranal in the mature virion. First, thin section analysis shows that the viral RNP in mature (and even released) virions which have been diametrally sectioned remains tightly associated with the underside of the viral envelope and does not appear to assume a more central location. Second, shortened proteins equivalent to the size of the 50,000 dalton protein produced from chymotrypsin treated vesicles are not isolated under normal conditions from infected cells or virions. Consequently, it is likely that the (carboxyl?) terminus remains in this peripheral location which comes to reside in the interior of the virion after maturation, and therefore would remain accessible to viral RNP.

These processes produce a mature particle which then resides in smooth membrane (probably Golgi) vesicles. Release of virus is then accomplished, without cell lysis, by the exocytosis of these vesicles at the plasma membrane, by processes similar or identical to the release of secretory proteins by Golgi vesicles. This process does not appear to leave appreciable amounts of viral antigen in the plasma membrane. Therefore, either incorporation of viral glycoproteins present in vesicular membranes into virion is highly efficient, or possibly, residual glycoprotein is shed into the media in the form of small micellar structures which we have shown can be antibody-linked to released virus present on cell surfaces.

VIII. Experiments to be Pursued or Completed During the Remainder of the Current Contract Year.

The data and methodology described above will serve as a basis for the continuation or initiation of the following studies, most of which can realistically be expected to be completed in the current contract year. Other experiments (noted below) which will be dependent upon successful completions of other studies or which are closely related to studies proposed for the following year (see renewal proposal) will be extended into the next year.

(1) Suitable methods have not yet been developed for the production and purification of large quantities of viral particles and virus-specific antigens - free from host cell contaminants. Although this is in part due to the generally low titers of virus produced from the cell cultures which have been used, the major problem seems to be that sandfly fever viruses are unstable in the procedures used for virus concentration and purification. This has been noted by other laboratories working with sandfly fever viruses (1, 2, 8), and seems to be a property of other bunyaviruses as well (7).

This, however, has not hindered the studies on an analytical scale of intracellular viral protein synthesis, due to the immunoprecipitation procedures which we have developed. As indicated in this report, these procedures have been used to approach, indirectly, many other aspects of sandfly fever virus replication. However, the difficulties in virus particle purification has impeded the production of preparative amounts of viral antigens necessary for animal protection studies. In addition, the purification problem complicates the rigorous identification of the structural proteins of sandfly fever viruses, which is critical as it serves a baseline for most other studies relating to viral replication and experimental immunogen production. Alternate procedures for the isolation of suitable amounts of viral antigens are possible, and some are suggested below, but they assume a firm knowledge of the relevant structural proteins.

We have shown that the direct immunoprecipitation of ^{3}H-leucine-labelled Karimibad virus particles gives preparations free of contaminating host cell debris, makes no assumptions of antibody activity to all structural proteins, avoids the problems of virus instability in density gradients, and can be conveniently and consistently accomplished with the virus yields from 10^{7} infected cells or less. Therefore, we will use these procedures to rigorously identify the structural proteins and glycoproteins of Naples, Sicilian, Chagres, Itaporanga, Candiru and others noted below.

(2) The experiments reported have indicated that the inhibition of protease activity by zinc may reveal "polycistronic" precursor proteins, which if reproducible, would suggest several central events occuring not only in the post-translational processing of virus proteins, but also in the manner in which viral transcripts are synthesized (see renewal proposal, section III-B). The preliminary results presented above have defined the concentrations of this inhibitor that will be used in this system, however, it is clear that optimal concentrations were not selected. Therefore, the effect on viral protein synthesis of several concentrations of zinc, intermediate between 0.05 mM and 1.0 mM, will be examined and at various times after infection. If this protein is consistently identified in immunoprecipitates and satisfies the criteria for viral specificity described in Section III-1, pulse-chase procedures will again be employed to attempt to identify precursor-product relationships. If pulse-chase procedures are not successful in these reduced concentrations of zinc, the relationship of this high molecular weight protein to other virus-specifc polypeptides will be determined by tryptic peptide mapping (see renewal proposal, Section III-A).

(3) In studies presented above, amino acid analogues and tunicamycin were used to inhibit post-translational processing of viral proteins. The former has suggested that minor cleavages occur in the maturation of viral glycoproteins,

and the latter was used to demonstrate that glycosylation of viral glycoproteins occurs exclusively at asparagine residues. However, in the appropriate concentrations, synthesis of the (altered) polypeptides was not diminished. From an analogy to other systems (discussed above), it is possib that virus particles which may have very low specific infectivities are nonetheless assembled. Either these particles or mutants with defined lesions at cleavage sites (59) may be of potential value in immunization. Whether virus particles are produced in the presence of these compounds could be assayed rapidly by thin-section electron microscopy. Such studies would be of value even if particles are not produced. The normal events which occur in morphogenesis are now known - at least on an ultrastructural level. Since the effect of these inhibitors has also been determined on a molecular level, an inhibitor-induced arrest of morphogenesis at a particular stage in assembly could be useful in determining the function of specific viral proteins in this process.

(4) Our studies, which have characterized the glycosylation patterns of sandfly fever viruses, have demonstrated that they contain only polymannose glycopeptides. Consequently, they lack sialic acid, galactose, fucose, and peripheral N-acetylglucosamine. Although the reason for this unusual pattern is not clear, this data may be useful in designing purification methods for viruses and viral antigens. As the activity of various lectins is specific for certain sugar residues or groups of sugar residues (48), affinity chromatography using the appropriate lectins bound to agarose beads could be used to select viral glycoproteins or particles, or alternatively, to remove non-viral material. Specifically, castor bean lectin, Ricin D, Ricin I, peanut lectin, and soybean lectin which bind to galactose or galactosamine; limulin, which binds to sialic acid; and ulex lectin (anti-H lectin) which binds to fucose, would not recognize viral glycoproteins or particles, but would remove most host-specific glycoproteins or membranes. The eluant from such affinity columns could then be applied to columns containing Convanavalin A or lentil lectin which recognize mannose residues. Non-glycosylated proteins could then be removed in buffer and viral glycoproteins eluted with methyl-D-glucopyranoside.

The advantage of these affinity columns lies not only with their specificity but also in their enormous concentrating ability. Several liters may be passed through a 2.0 cm x 20 cm column, and the proteins specifically eluted into 10-20 mls. We will evaluate such lectin columns for their ability to purify and concentrate viral antigens and particles. Although the stability of virus particles in these procedures is unknown, viral glycoproteins should be obtained irrespective of particle stability, and would be suitable for the animal vaccination studies previously proposed (1979 proposal). The production of antigens based on immuno-affinity columns is discussed in the accompanying proposal.

(5) The analysis of immunoprecipitates by SDS gel electrophoresis has been found to be suitable for most experments, however, several questions (listed below) have not been resolved by this one-dimensional technique.

(a) By analogy to other bunyaviruses, it would be expected that sandfly fever viruses would possess two glycoproteins. However, in most sandfly fever viruses only one diffuse glycoprotein band is obtained. Although two bands are usually obtained from Naples and Punta Toro viruses, tryptic peptide analysis has not been done to demonstrate that these bands contain unique amino acid

sequences. At present it is assumed, but not proven, that sandfly fever viruses each possess two distinct membrane proteins.

(b) In immunoprecipitates from infected or uninfected cells, a polypeptide species of 43,000 daltons is consistently observed. Although this band is likely to represent, at least in part, the normal cell protein, actin, this band consistently contains more activity from infected than uninfected cells. One explanation of this observation is that two distinct polypeptides, one of viral and one of host origin, migrate to this position due to similar or identical molecular weight characteristics.

(c) In several sandfly viruses, but most notably Punta Toro virus, the capsid protein appears to be bracketed by bands of slightly higher and slightly lower molecular weights. These bands are incompletely resolved due to their similar molecular weights and the much greater amount of radioactivity in the capsid polypeptide. Consequently, it is unclear if these bands represent distinct virus proteins, although it is of interest that the upper band co-migrates with the Karimibad structural proteins, NVP-29.

(d) Assuming that the molecular weights of the Punta Toro and Karimibad virus genome segments, which were determined under non-denaturing conditions by Robeson, et al (1) are valid, the theoretical coding capability of these genomes is greatly in excess of that needed to code for the currently recognized structural and non-structural proteins. Although it is possible that some proteins are synthesized in very small amounts or are poorly immunogenic and thus are not detectable by these methods, it is also possible that some virus-specific proteins are immunoprecipitated but not observed because they co-migrate with other proteins.

It is likely that all of these questions could be resolved if immunoprecipitates were subjected to two-dimensional gel analysis, in which proteins were separated in the first dimension by isoelectric focusing, and in the second dimension by SDS gel electrophoresis. Although this laboratory routinely analyzes lysates from labelled rickettsia on such two-dimensional gels, technical problems have prevented the similar analysis of immunoprecipitates. Specifically, it has not been possible to completely and irreversibly solubilize immune precipitates by procedures compatible with isoelectric focusing (low salt and neutral pH). However, we have recently found that this can be accomplished by 2% triton X-100, 8 M urea, and 5% mercaptoethanol. Therefore, the questions referred to above can now be rapidly addressed by the much greater resolving ability of two-dimensional gels.

(6) In the study described above in which anti-viral antibody was added to the media of Punta Toro virus infected cells, it was observed that electron-dense particles could be linked to the surface of mature viral particles by antibody. Consequently, these smaller particles contain antigenic determinants in common with virus surface proteins. Their nature is unknown, but is was suggested that they may be either ribosomes from disrupted cells retaining nascent viral proteins, or alternatively, protein-lipid micelles shed from infected cells

or disaggregating virions. The observed electron density of these particles would then be a function of either the uranyl acetate staining of RNA or of the osmium staining of lipid. We will determine if these structures represent ribosomes by repeating the experiment as described above except that the monolayers will be treated with pancreatic ribonuclease prior to fixation and embedding. If these structures are resistant to ribonuclease it is likely that they represent a micellar arrangement of viral glycoproteins and lipid and may be analogous to the slowly sedimenting hemagglutinin released from flavivirus infected cells (62, 63, 64). Such a micellar structure would be expected to be an active immunogen (see 1979 proposal), and hence, attempts would be made to determine if such structures appear in appreciable quantities in media from infected cultures, and if they can be isolated on density gradients.

(7) One obvious application of the immunoprecipitation procedures which we have used is to perform cross immunoprecipitations using labelled lysates from cells infected by one virus and HMAF raised against another virus. Such studies would not only indicate the presence of serological cross reactivity, but also the polypeptide which contained the cross-reacting determinants. A comparison of the gel profiles from such cross immunoprecipitations with other known serological cross reactions could indicate which functions are carried out by which viral proteins.

For example, Tesh has shown that Karimibad and Punta Toro viruses are not cross reactive in neutralization tests but do cross react by hemagglutination inhibition tests (71). We have carried out very recently cross-immunoprecipitation reactions using labelled lysates from Punta Toro and Karimibad virus-infected cells and HMAF specific for Punta Toro or Karimibad, or rabbit antisera specific for Rift Valley Fever Virus (obtained from Dr. J. Gibbs, NIH). As can be seen in figure 23, Karimibad HMAF precipitated the smaller Punta Toro glycoprotein much more efficiently than the larger. The reciprocal experiment is consistent with the interpretation that Punta Toro HMAF efficiently precipitated only one of the Karimibad glycoproteins (which are not resolved by these procedures). Therefore, this experiment suggests 1) that two glycoprotein bands of Punta Toro, in fact, contain distinct antigenic determinants, 2) that the smaller Punta Toro virus glycoprotein may be responsible for hemagglutination (since these viruses cross react by HI), and 3) that the larger Punta Toro glycoprotein may be responsible for virus neutralization, since it is not efficiently precipitated with Karimibad HMAF, these viruses do not cross react by neutralization tests, and it is the only other viral surface antigen. This hypothesis will be tested by the cross-immunoprecipitation of other virus pairs. It is also clear that despite the differences in molecular weight, shared antigenic determinants also appear to exist on the nucleocapsid proteins. The cross reactivity demonstrated by the RVF antisera requires further study.

Cross immunoprecipitation could also be used in other studies, notably the analysis of recombinant strains produced by genome segment reassortment. Such an analysis would be much quicker than oligonucleotide mapping, would require very little material for analysis, and the analysis of several cloned recombinants

could indicate which genome segments produce structural and non-structural proteins (by linkage). Cross-immunoprecipitations of a large number of sandfly fever virus pairs could indicate whether or not segment reassortment has occurred in nature. Although such a large-scale analysis is not currently planned, one such pair would be of special interest. Gordil and Saint Floris viruses were isolated in 1974 (72) from the same animal (a gerbil). Therefore, in studies which are planned in collaboration with Dr. C. J. Peters (USAMRIID), cross immunoprecipitation will be carried out between these two viruses (as described above). The results will be analyzed to determine if these viruses possess similar proteins in patterns consistent with the coding specificities of genome segments established for the California group viruses (3).

(8) In studies reported above, we have shown that membranes from infected cells can be isolated, transformed into vesicles, and examined to determine which viral proteins are membrane-bound and the relative orientation of the viral proteins in these membranes. Since sandfly viruses mature (bud) at internal smooth membranes, it should be possible to describe very precisely the events which occur in this process and the relative roles of individual viral proteins. Although these studies will continue in the current contract year, most of this work will be carried out in the following year. Consequently these studies will be described in the accompanying proposal.

IX. Bibliography

1. Robeson, G., L. H. ElSaid, W. Brandt, J. Dalrymple and D. H. L. Bishop. 1979. Biochemical Studies on the Phlebotomus Fever Group Viruses (Bunyaviridae Family). J. Virol., in press.

2. Peters, C. J., USAMRIID, personal communication.

3. Bishop, D. H. L. and R. E. Shope. Bunyaviridae. In Comprehensive Virology 14. Chapter I. (Plenum Press, New York, 1979).

4. Bishop, D. H. L. Genetic Potential of Bunyaviruses. In Current Topics in Microbiology and Immunology. 86: 1 - 33. (Springer-Verlag, Newton, 1979)

5. Murphy, F. A., A. K. Harrison, and S. G. Whitfield. 1973. Bunyaviridae: Morphologic and Morphogenetic Similarities of Bunyamwera Supergroup Viruses and Several Other Arthropod-Borne Viruses. Intervirology, 1: 297 -316.

6. Darwish, M. A. and I. Z. Imam. 1979. Arthropod-Borne Virus Information Exchange. March, p. 76-82.

7. Lazdins, I. and I. H. Holmes. 1979. Protein Synthesis in Bunyamwera Virus-Infected Cells. J. Gen. Virol., 44: 123-133.

8. Harrison, S. A., J. M. Dalrymple, and W. E. Brandt, WRAIR, person communication.

9. David-West, T. S. 1974. Polyacrylamide Gel Electrophoresis of Dugbe Virus Infected Cells. Microbias 11, 21-23.

10. Vezza, A. C., P. Repik, P. Cash and D. H. L. Bishop. 1979. In Vivo Transcription and Protein Synthesis Capabilities of Bynyaviruses: Wild-Type Snowshoe Hare Virus and Its Temperature-Sensitive Group I, Group II and Group I/II Mutants. J. Virol., 31: 426 - 436.

11. Wirth, D. F., F. Katz, B. Small and H. F. Lodish. 1977. How a Single Sindbis Virus mRNA Directs the Synthesis of One Soluble Protein and Two Integral Membrane Glycoproteins. Cell, 10: 253 - 263.

12. Laemmli, Ul K. 1970. Cleavage of Structural Proteins During the Assembly of the Bacteriophage T4. Nature 227: 680 - 685.

13. Laskey, R. A., and A. D. Mills. 1975. Quantitative Film Detection of ^{3}H & ^{14}C in Polyacrylamide Gels by Fluorography. Eur. J. Biochem., 56: 335 - 341.

14. Gentsch, J. and D. H. L. Bishop. 1978. The Small Viral RNA Segment of Bunyaviruses Codes for the Viral Nucleocapsid (N) Protein. J. Virol., 28: 417.

15. Gentsch, J., D. H. L. Bishop and J. F. Obijeski. 1977. The Virus Particle Nucleic Acids and Proteins of Four Bynyaviruses. J. Virol., 34: 257.

16. Butterworth, B. E. 1976. Proteolytic Processing of Animal Virus Proteins. Current Topics in Microbiol. and Immunology, 77: 2 - 41.

17. Aliperti, G. and M. J. Schlesinger. 1978. Evidence for an Autoprotease Activity of Sinbis Virus Capsid Protein. Virology, 90: 366 - 369.

18. Kaempfer, R. and M. Meselson. 1969. Studies of Ribosomal Subunit Exchange. Cold Spring Harbor Sumposia on Quantitative Biology, vol. 34: 209 - 230.

19. Smith, J. F., and D. T. Brown. 1977. Envelopment of Sindbis virus: Synthesis and Organization of Proteins in Cells Infected With Wild Type and Maturation-Defective Mutants. J. Virol., 22: 662 - 678.

20. Pfefferkorn, E. R. and D. Shapiro. Reproduction of Togaviruses. In Comprehensive Virology, vol. 2: 171 - 220. 1974.

21. Bracha, M. and M. J. Schlesinger. 1976. Inhibition of Sindbis Virus Replication by Zinc Ions. Virology, 72: 272 - 277.

22. Hynes, R. O., J. A. Wyke, J. M. Bye, K. C. Humphreys and E. S. Pearlstein. In Proteases and Biological Control (E. Reich, D. B. Rifkin and E. Shaw, ed) pp. 931 - 944. (Cold Spring Harbor Lab, Cold Spring Harbor, New York, 1975).

23. Blumberg, D. M. and P. W. Robbins. In Proteases and Biological Control (E. Reich, D. B. Rifkin and E. Shaw, ed) pp. 945 - 956. (Cold Spring Harbor Lab, Cold Spring Harbor, New York, 1975)

24. Kaluza, G. 1976. Early Sunthesis of Semliki Forest Virus Proteins in Infected Chicken Cells. J. Virol., 19: 1 - 12.

25. Massey, R. J. and G. Schochetman. 1979. Gene Order of Mouse Mammary Tumor Virus Precursor Polyproteins and Their Interaction Leading to the Formation of a Virus. Virology, 99: 358 - 371.

26. Buchmeier, M. J., M. B. A. Oldstone. 1979. Protein Structure of Lymphocytic Choriomeningitis Virus: Evidence for a Cell-Associated Precursor of the Virion Glycopeptides. Virology, 99: 111 - 120.

27. Noel, D., K. Nikaido and G. F. L. Ames. 1979. A Single Amino Acid Substitution in a Histidine-Transport Protein Drastically Alters Its Mobility in Sodium Dodecyl Sulfate-Polyacrilimide Gel Electrophoresis. Biochem., 18: 4159 - 4165.

28. Blobel, G. 1976-1977. Synthesis and Segregation of Secretory Proteins: The Signal Hypothesis. Inter. Cell Bio., 318 - 325.

29. Sabin, A. B., C. B. Philip and J. R. Paul. 1944. Phlebotomus (Pappataci or Sandfly) Fever. A Disease of Military Importance. Summary of Existing Knowledge and Preliminary Report of Original Investigations. J. Am. Med. Assoc., 125: 693 - 699.

30. Sabin, A. B. 1955. Recent Advances in Our Knowledge of Dengue and Sandfly Fever. Amer. J. Tropical Medicine and Hygiene, 4: 198 - 207.

31. Palmiter, R. D., S. N. Thibodcav, J. Gagnon, and K. A. Walsh. 1978. Role of Proteases in the Secretion of Lysozyme, Ovomucoid, Conalbumin and Ovalbumin from the Chick Oviduct. Fed. Eur. Biochem. Soc. Proc. Meet., 47: 89 - 101.

32. Nagai, Y. and H. D. Klenk. 1977. Activation of Precursers to Both Glycoproteins of Newcastle Disease Virus by Proteolytic Cleavage. Virology, 77: 125 - 134.

33. Scheid, A., M. C. Graves, S. M. Silver and P. W. Choppin. Studies on the Structure and Functions of Paramyxovirus Glycoproteins. In Negative Strand Viruses and the Host Cell. (Mahy and Barry, ed). (Academic Press, New York, 1978).

34. Holmes, I. H. 1971. Morphological Similarity of Bunyamwera Supergroup Viruses. Virology, 43: 708: 708 - 712.

35. Duda, E. and M. J. Schlesinger. 1975. Alterations in Sindbis Viral Envelope Proteins by Treating Bltk cells with Glucosamine. J. Virol, 15: 416 - 419.

36. Learitt, R., S. Schlesinger and S. Kornfeld. 1977. Tunicamycin Inhibits Glycosylation and Multiplication of Sindbis and Vesicular Stomatitis Viruses. J. Virol., 21: 375 - 385.

37. Schwarz, R. T., J. R. Rohrschneider and M. F. G. Schmidt. 1976. Suppression of Glycoprotein Formation of Semliki Forest, Influenza and Avian Sarcoma Virus by Tunicamycin. J. Virol., 19: 782 - 791.

38. Gibson, R., R. Learitt, S. Kornfeld and S. Schlesinger. 1978. Synthesis and Infectivity of Vesicular Stomatitis Virus Containing Nonglycosylated G Protein. Cell, 13: 671 - 679.

39. Courtney, R. J. 1976. Herpes Simplex Virus Protein Synthesis in the Presence of 2-Deoxy-D-Glucose. Virology, 73: 286 - 294.

40. Leavitt, R., S. Schlesinger and S. Kornfeld. 1977. Impaired Intracellular Migration and Altered Solubility of Nonglycolylated Glycoproteins of Vesicular Stomatitis Virus and Sindbis Virus. J. Bio. Chem., 252: 9018 -9023.

41. Muller, W. E. G. Mechanisms of Action and Pharmacology: Chemical Agent. In Antiviral Agents and Viral Diseases of Man (G. J. Galasso, T. C. Merigan and R. A. Buchanan, ed.) Chap. 4: pp. 135. (Raven Press, New York, 1979).

42. Hubbard, A. L., G. Wilson, G. Ashwell and H. Stukenbrok. 1979. An Electron Microscope Autoradiographic Study of the Carbohydrate Recognition Systems in Rat Liver (I. Distribution of ^{125}I-Ligands among the Liver Cell Types). J. Cell Bio., 83: 47 - 64.

43. Hubbard. A. L. and H. Stukenbrok. 1979. An Electron Microscope Autoradiographic Study of the Carbohydrate Recognition Systems in Rat Liver (II. Intracellular Fates of the ^{125}I-Ligands). J. Cell Bio., 83: 65 - 81.

44. Jahrling, P.B. and J.L. Beall. 1977. Chromatographic Separations of Alphavirus Strains by Hydroxylapatite. J. Clin. Micro. 6: 238-243.

45. Jahrling, P.B. and L. Gorelkin. 1975. Selective Clearance of a Benign Clone of Venezuelan Equine Encephalitis Virus from Hamster Plasma by Hepatic Reticuloendothelial Cells. J. Infec. Dis. 132: 667-676.

46. Jahrling, P.B., G.B. Heisey and R.A. Hesse. 1977. Evaluation of Vascular Clearance as a Marker for Virulence of Alphaviruses: Disassociation of Rapid Clearance with Low Virulence of Venezuelan Encephalitis Virus Strains in Guinea Pigs. Inf. Immun. 17: 356-360.

47. Kemp, M.C., S. Basak and R.W. Campans. 1979. Glycopeptides of Murine Leukemia Viruses (I. Comparison of Two Ecotropic Viruses.). J. Virol. 31: 1-7.

48. Dulaney, J.T. 1979. Binding Interactions of Glycoproteins with Lectins. Mol. Cell. Biochem. 21: 43-63.

49. Hughes, R.C. Membrane Glycoproteins, Chapter 6. (Butterworth and Company, Boston, 1975).

50. Turco, S.J. and P.W. Robbins. 1978. Synthesis and Function of Carbohydrate Units of Cell Surface Glycoproteins. In Fibroblast Surface Protein (A. Vaheri, E. Ruoslahti, and D.F. Mosher, ed.) pp. 392-398. (The New York Academy of Sciences, New York, 1978).

51. Sefton, B.M. 1977. Immediate Glycosylation of Sindbis Virus Membrane Proteins. Cell 10: 659-668.

52. Keil, W., H.D. Klenk and R.T. Schwarz. 1979. Carbohydrates of Influenza Virus (III. Nature of Oligosaccharide-Protein Linkage in Viral Glycoproteins). Virology 31: 253-256.

53. Tabas, I., S. Schlesinger and S. Kornfeld. 1978. Processing of High Mannose Oligosaccharides to Form Complex Type Oligosaccharides on the Newly Synthesized Polypeptides of the Vesicular Stomatitis Virus G Protein and the IgG Heavy Chain. J. Bio. Chem. 253: 716-722.

54. Waechter, C.J. and W.J. Lennartz. 1976. The Role of Polyprenol-linked Sugars in Glycoprotein Synthesis. Ann. Review Biochem. 45: 95-112.

55. Kornfeld, R. and S. Kornfeld. 1976. Comparative Aspects of Glycoprotein Structure. Ann. Review Biochem. 45: 217-238.

56. Robbins, P.W., S.C. Hubbard, S.J. Turco and D.F. Wirth. 1977. Proposal for a Common Oligosaccharide Intermediate in the Synthesis of Membrane Glycoproteins. Cell 12: 893-900.

57. Harford, J.B. and C.J. Waechter. 1977. Effect of Exogenous Dolichyl Monophosphate on a Developmental Change in Mannosylphosphoryldolichol Biosynthesis. Biochem. and Biophys. Res. Comm. 76, No. 4.

58. Tamm, I. and P.B. Sehgal. 1978. Halobenzimidazole Ribosides and RNA Synthesis of Cells and Viruses. Adv. Virol. Res. 22: 187.

59. Scheid, A. and P.W. Choppin. In Proteases and Biological Control (E. Reich, D.B. Rifkin and E. Shaw, ed.) pp. 645-660. (Cold Spring Harbor Lab, Cold Spring Harbor, New York, 1975).

60. Nagai, Y., H.D. Klenk and R. Rott. 1976. Proteolytic Cleavage of the Viral Glycoproteins and Its Significance for the Virulence of New Castle Disease Virus. Virology 72: 494-508.

61. Jones, K.J., R.K. Scopham, J.A. Pfeil, K. Wan, B.P. Sagik and H.R. Bose. 1977. Interaction of Sindbis Virus Glycoproteins During Morphogenesis. J. Virol. 21: 778-787.

62. Smith, T.J., W.E. Brandt, J.L. Swanson, J.M. McCown and E.L. Buescher. 1970. Physical and Biological Properties of Dengue-2 Virus and Associated Antigens. J. Virol. 5: 524-532.

63. Cardiff, R.D., W.E. Brandt, T.G. McCloud, D. Shapiro and P.K. Russell. 1971. Immunological and Biophysical Separation of Dengue-2 Antigens. J. Virol. 7: 15-23.

64. Brandt, W.E., D. Chiewsilp, D.L. Harris and P.K. Russell. 1970. Partial Purification and Characterization of a Dengue Virus Soluble Complement-Fixing Antigen. J. Immunol. 105: 1565.

65. Petri, T., H. Meier-Ewert and R.W. Compans. 1979. Inhibition of Influenza C Virus Replication by Actinomycin D, α -Amanitin, and UV Irradiation. J. of Virol. 32: 1037-1040.

66. Pringle, C.R., Enucleation as a Technique in the Study of Virus-Host Interactions. In Current Topics in Microbiology and Immunology 76 (Springer-Verlag, New York, 1978).

67. Maeno, K., S. Yoshii, K. Mita, M. Hamaguchi, T. Yoshida, M. IINuma, Y. Nagai and T. Matsumoto. 1979. Analysis of the Inhibitory Effect of Canavanine on the Replication of Influenza RI/5$^+$ Virus. Virology 94: 128-137.

68. Chatis, A. and T.G. Morrison. 1979. Vesicular Stomatitis Virus Glycoprotein Is Anchored to Intracellular Membranes Near Its Carboxyl End and Its Proteolytically Cleaved at Its Amino Terminus. J. Virol. 29: 957-963.

69. Nicolson, G.L. and R.G. Painter. 1973. Anionic sites of human erythrocyte membranes. II Antispectrin-reduced binding sites for positively charged colloidal particles. J. Cell Biol. 59: 395-406.

70. Walsh, F.S., B.H. Barber and M.J. Crumpton. 1976. Preparation of Inside-out Vesicles of Pig Lymphocyte Plasma Membrane. Biochem. 15: 3557-3563.

71. Tesh, R., P. Peralta, R. Shope, B.N. Chaniotis, and K.M. Johnson. 1975. Antigenic Relationships Among Phlebotomus Fever Group Arboviruses and Their Implications for the Epidemiology of Sandfly Fever. Am. J. Trop. Med. Hyg. 24: 135-144.

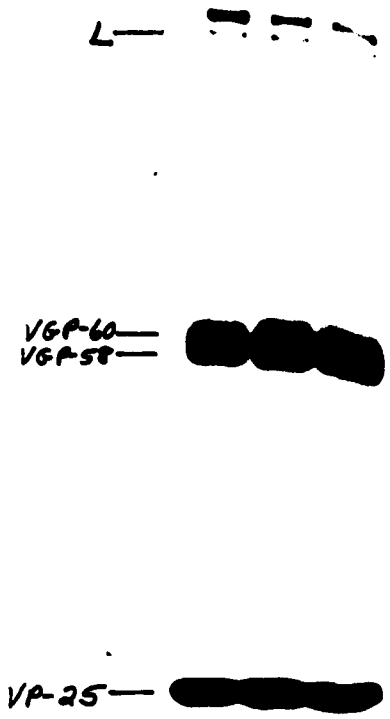


FIGURE 1

STRUCTURAL PROTEINS OF KARIMIBAD VIRUS

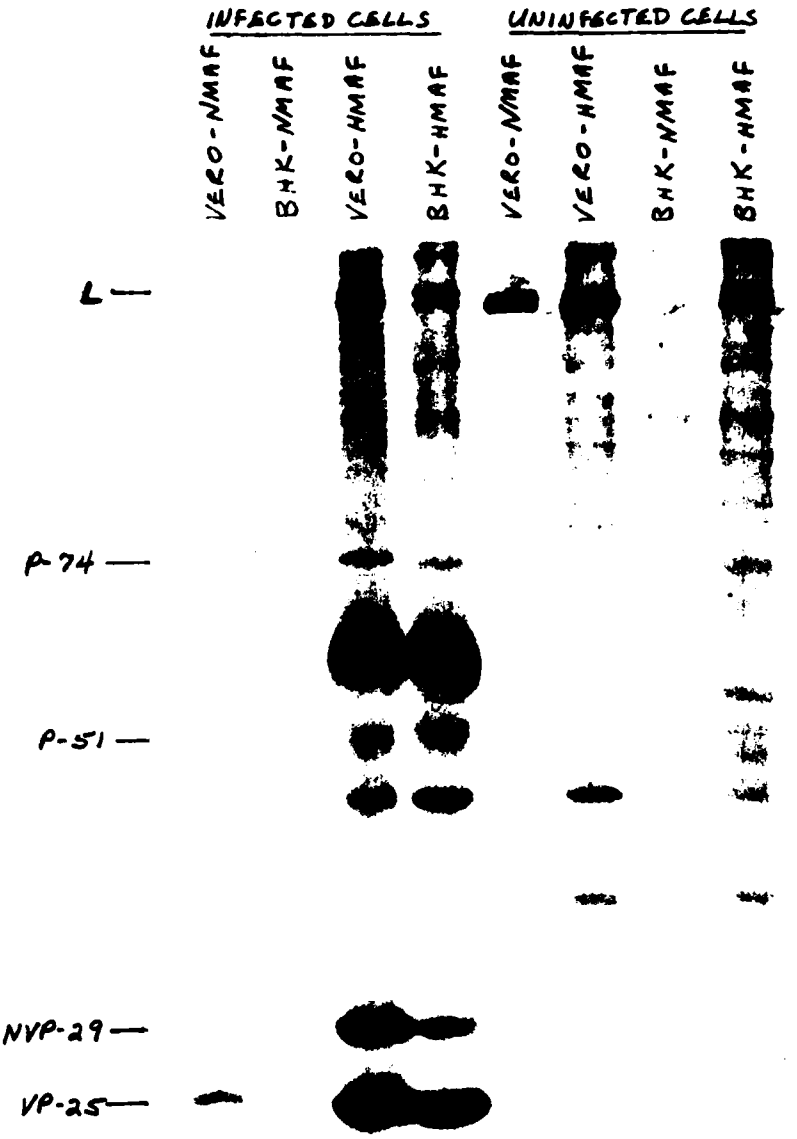


FIGURE 2

IMMUNOPRECIPITATION OF KARIMIBAD VIRUS-INFECTED CELLS AND UNINFECTED CELLS USING HYPERIMMUNE AND NORMAL ASCITIC FLUIDS

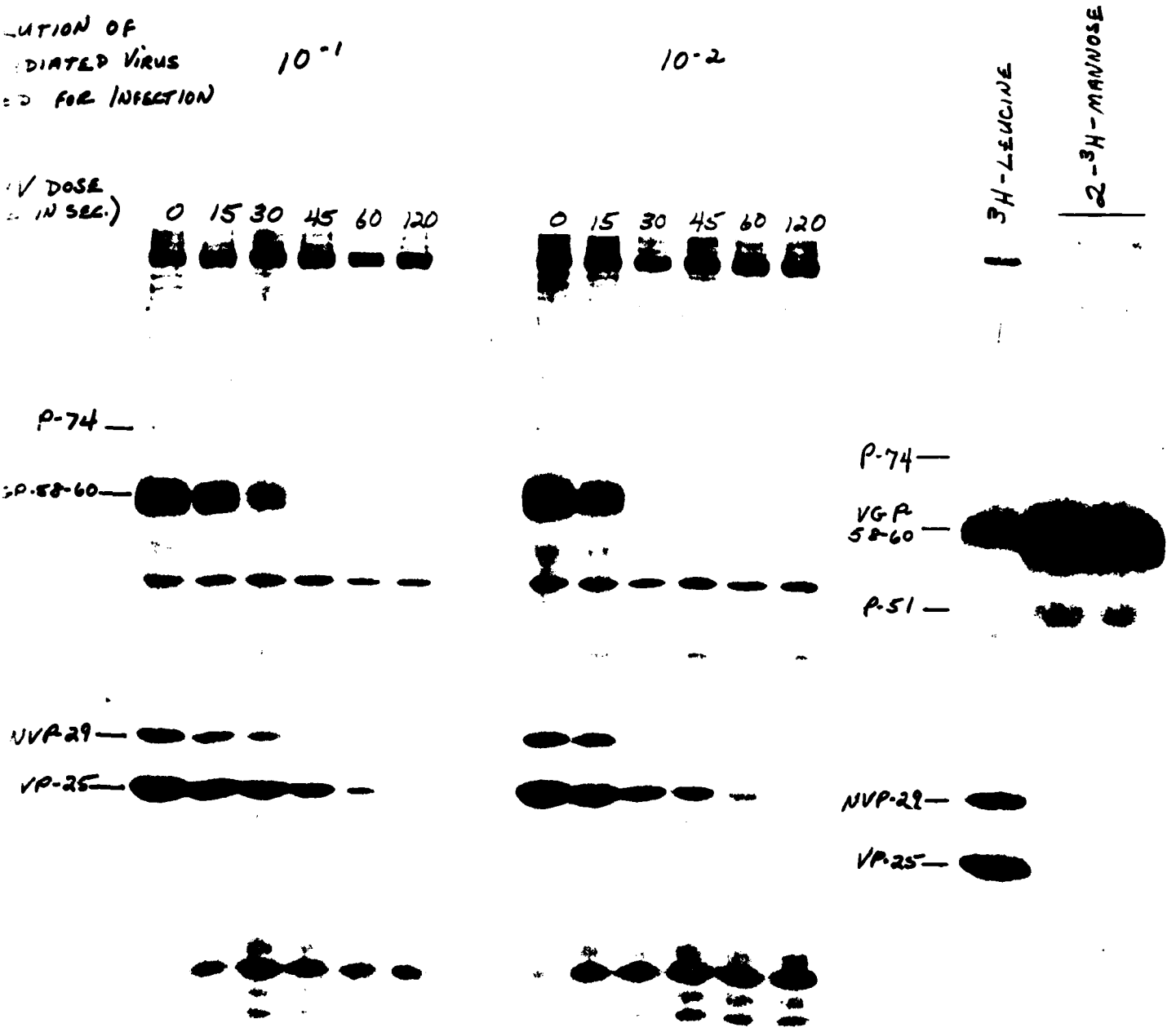


FIGURE 3

UV IRRADIATION OF KARIMIBAD VIRUS PRIOR TO INFECTION

FIGURE 4

IMMUNOPRECIPITATION OF ³H-LEUCINE OR 2-³H-MANNOSE LABELLED KARIMIBAD VIRUS PROTEINS

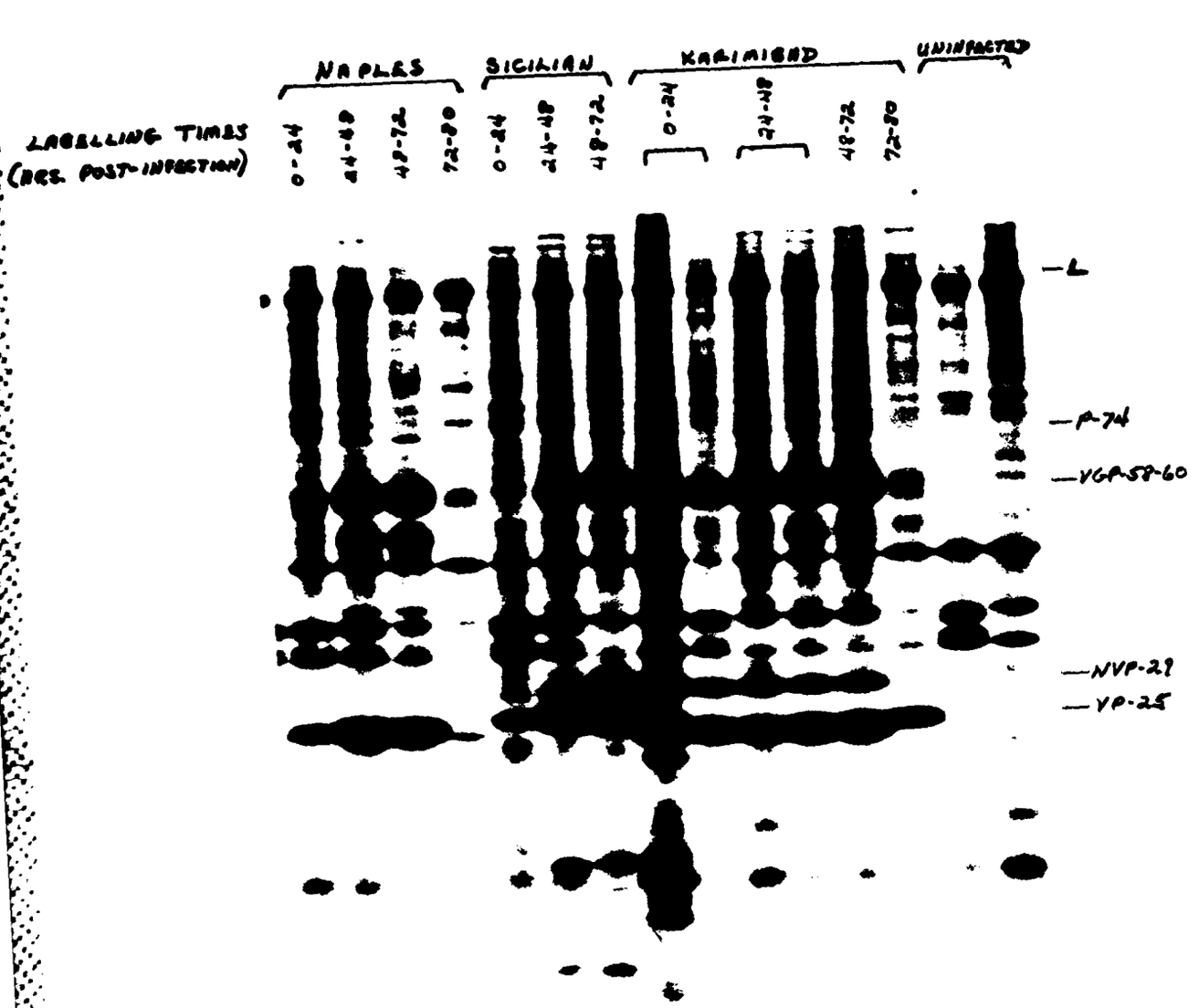


FIGURE 5

IMMUNOPRECIPITATIONS FROM NAPLES, SICILIAN, OR KARIMABAD VIRUS-INFECTED CELLS USING HOMOLOGOUS HMAF. CULTURES WERE LABELLED AT THE TIMES INDICATED. THE KARIMABAD VIRUS-SPECIFIC PROTEINS ARE MARKED FOR ORIENTATION.

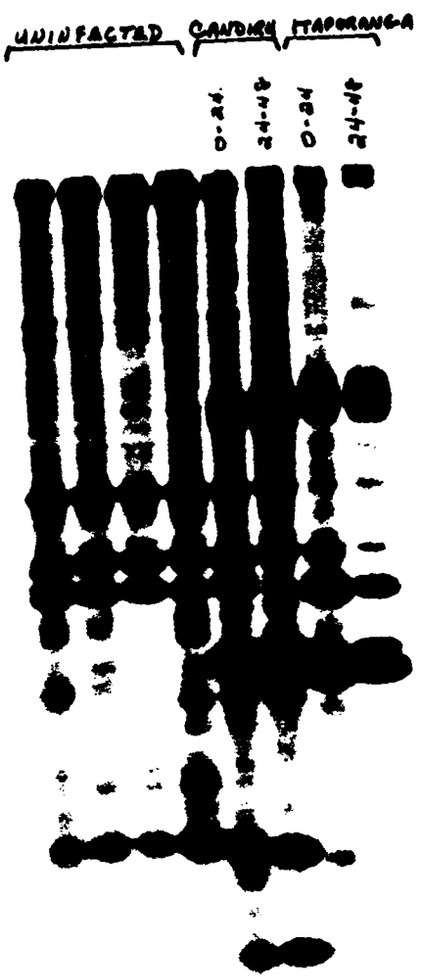


FIGURE 6

IMMUNOPRECIPITATIONS FROM CANDIRU AND ITAPORANGA VIRUS-INFECTED CELLS USING HOMOLOGOUS HMAF.

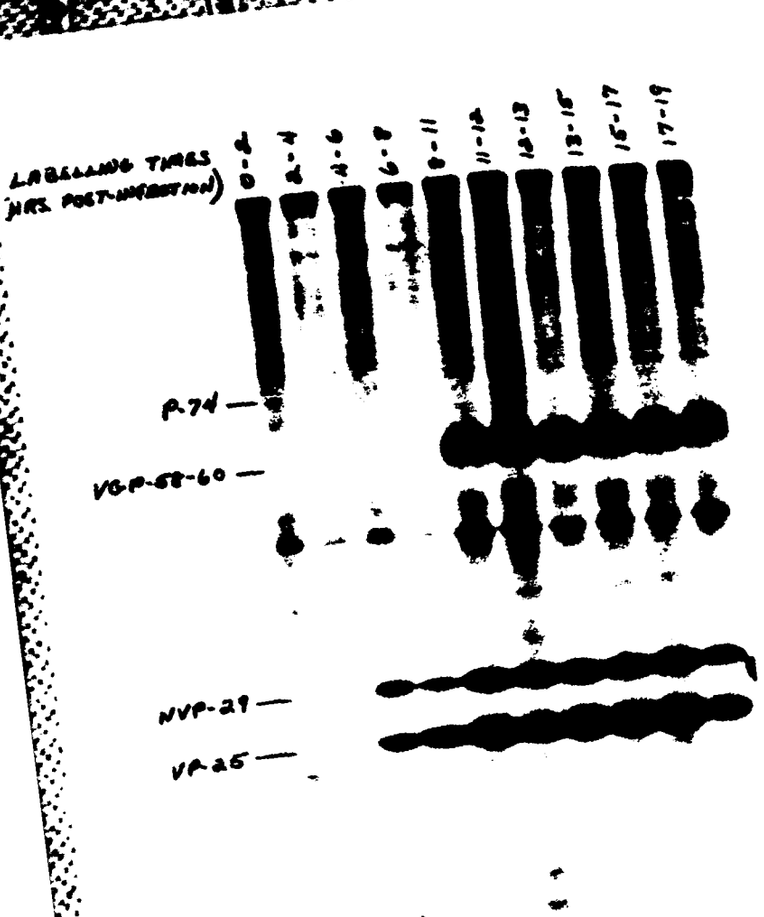


FIGURE 7

KINETICS OF KARIMBAD VIRUS-SPECIFIC PROTEIN SYNTHESIS

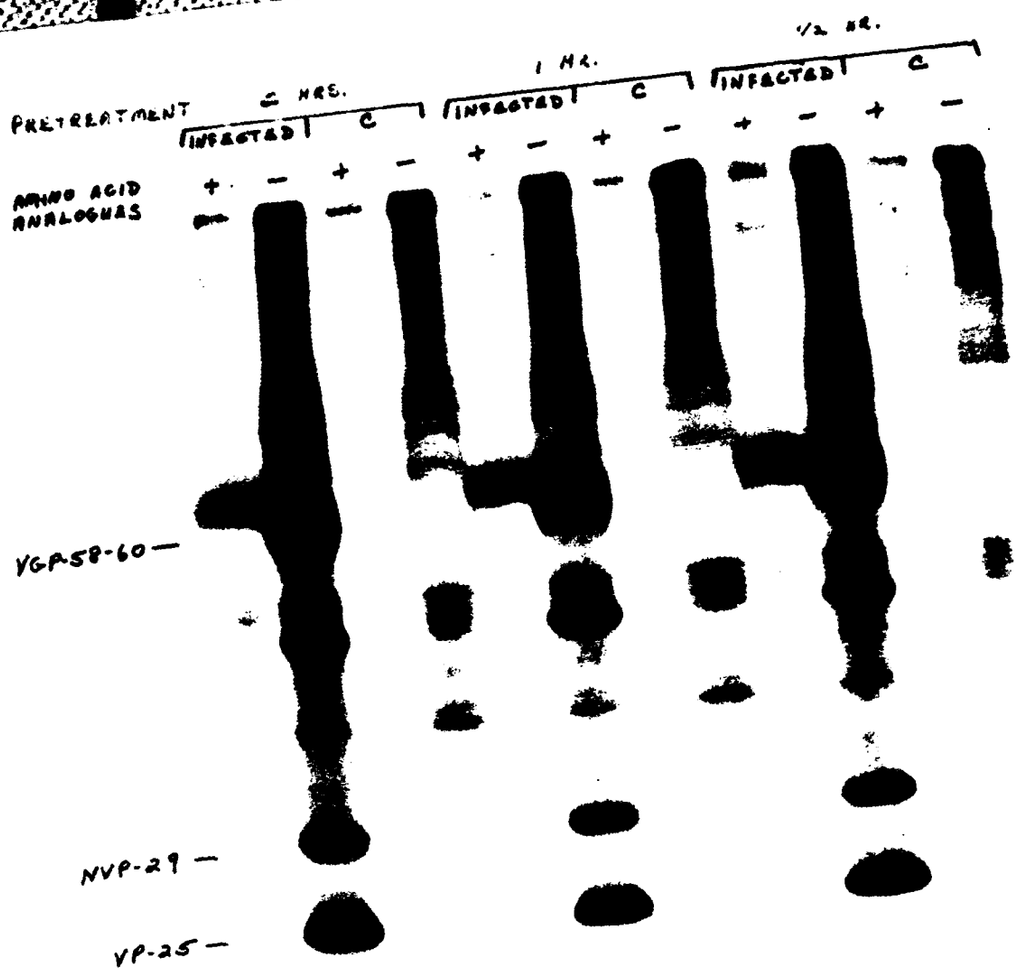
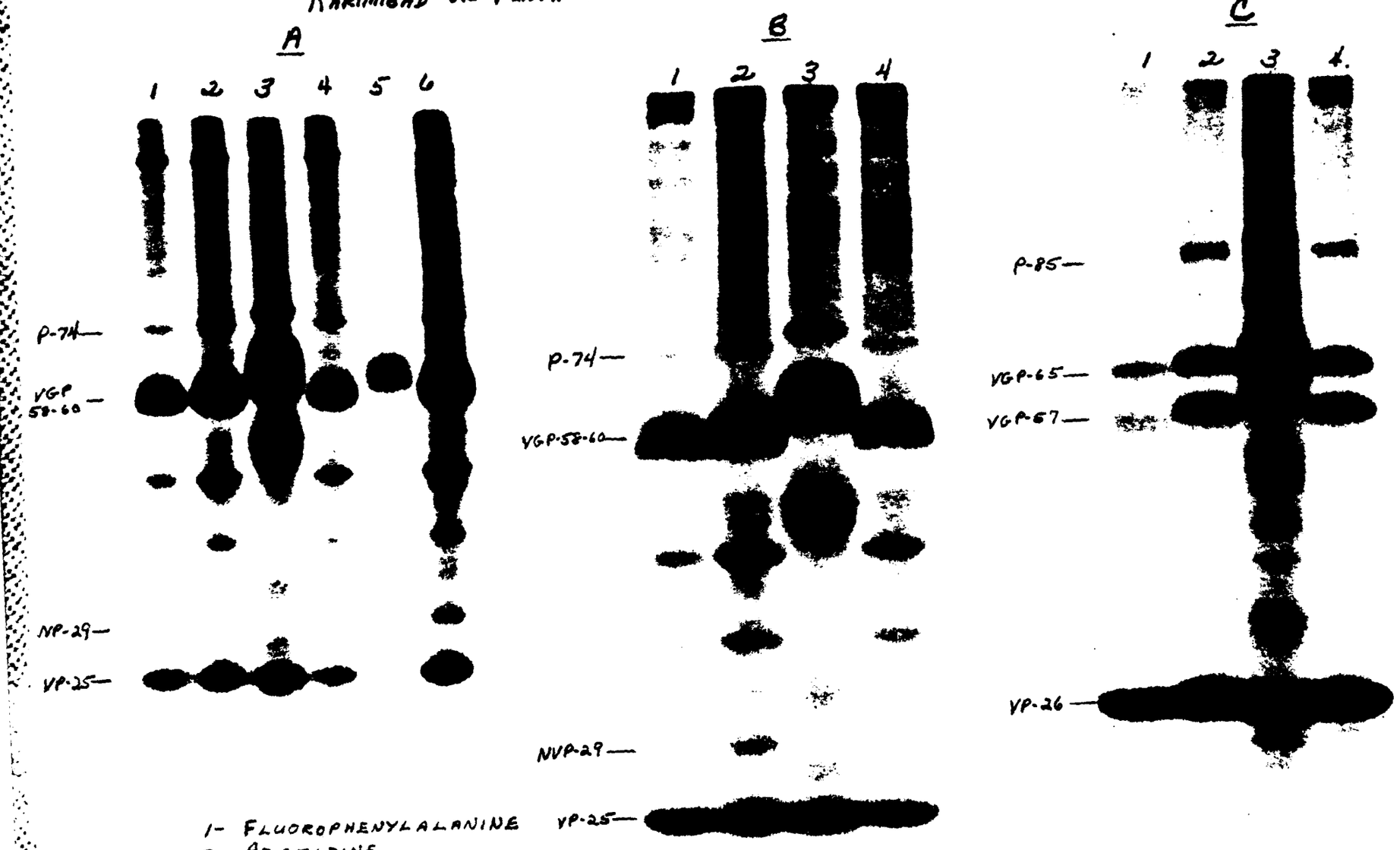


FIGURE 8

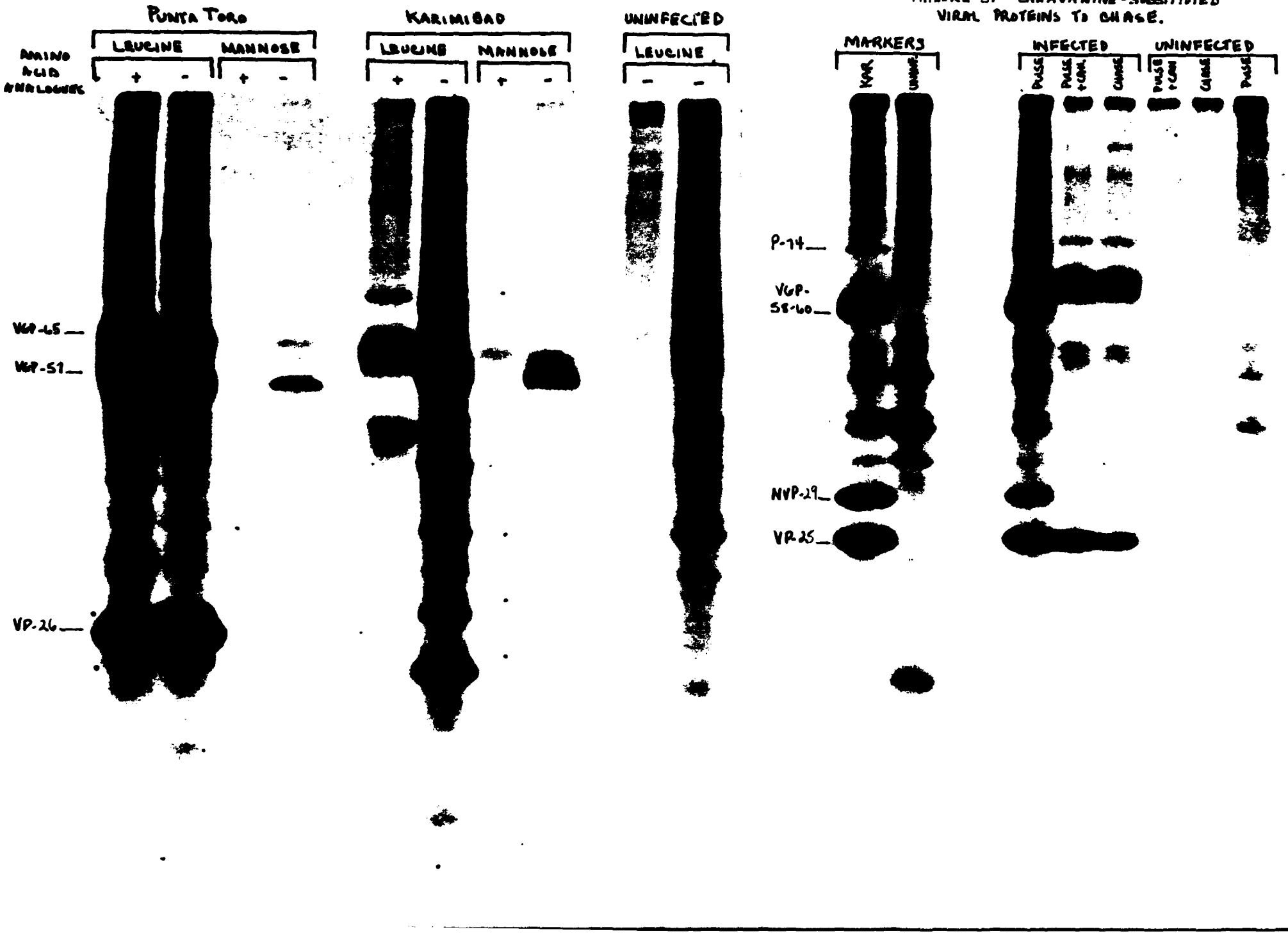
EFFECT OF AMINO ACID ANALOGUES ON KARIMBAD VIRUS-SPECIFIC PROTEIN SYNTHESIS

EFFECT OF INDIVIDUAL AMINO ACID ANALOGUES ON KARIMABAD OR PUNTA TORO VIRUS-SPECIFIC PROTEIN SYNTHESIS



1- FLUOROPHENYLALANINE
2- AZETIDINE
3- CANAVANINE
4- ETHIONINE

PAGE 5


FAILURE OF CANAVANINE-SUBSTITUTED VIRAL PROTEINS TO CHASE.
PUNTA TORO
KARIMABAD
UNINFECTED
LEUCINE
MANNOSE
AMINO ACID ANALOGUES
+
-
VGP-65
VGP-51
VP-26
MARKERS
KAR
UNINF.
INFECTED
UNINFECTED
PULSE
PULSE + CHASE
CHASE
P-74
VGP-58-60
NVP-29
VP-25

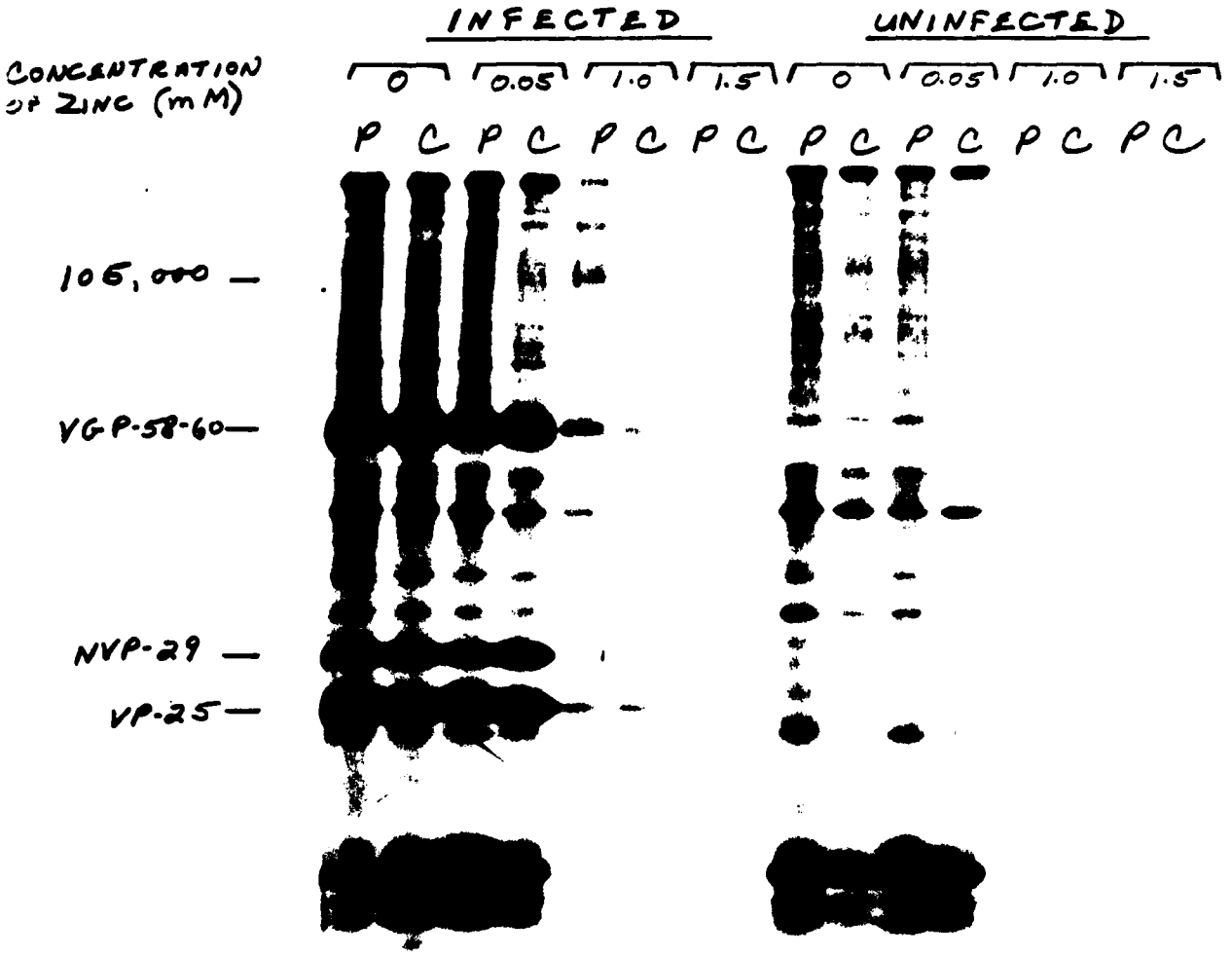


FIGURE 12

EFFECT OF ZINC IONS ON THE SYNTHESIS OF KARIMIBAD VIRUS-SPECIFIC PROTEINS (P=PULSE, C=CHASE)

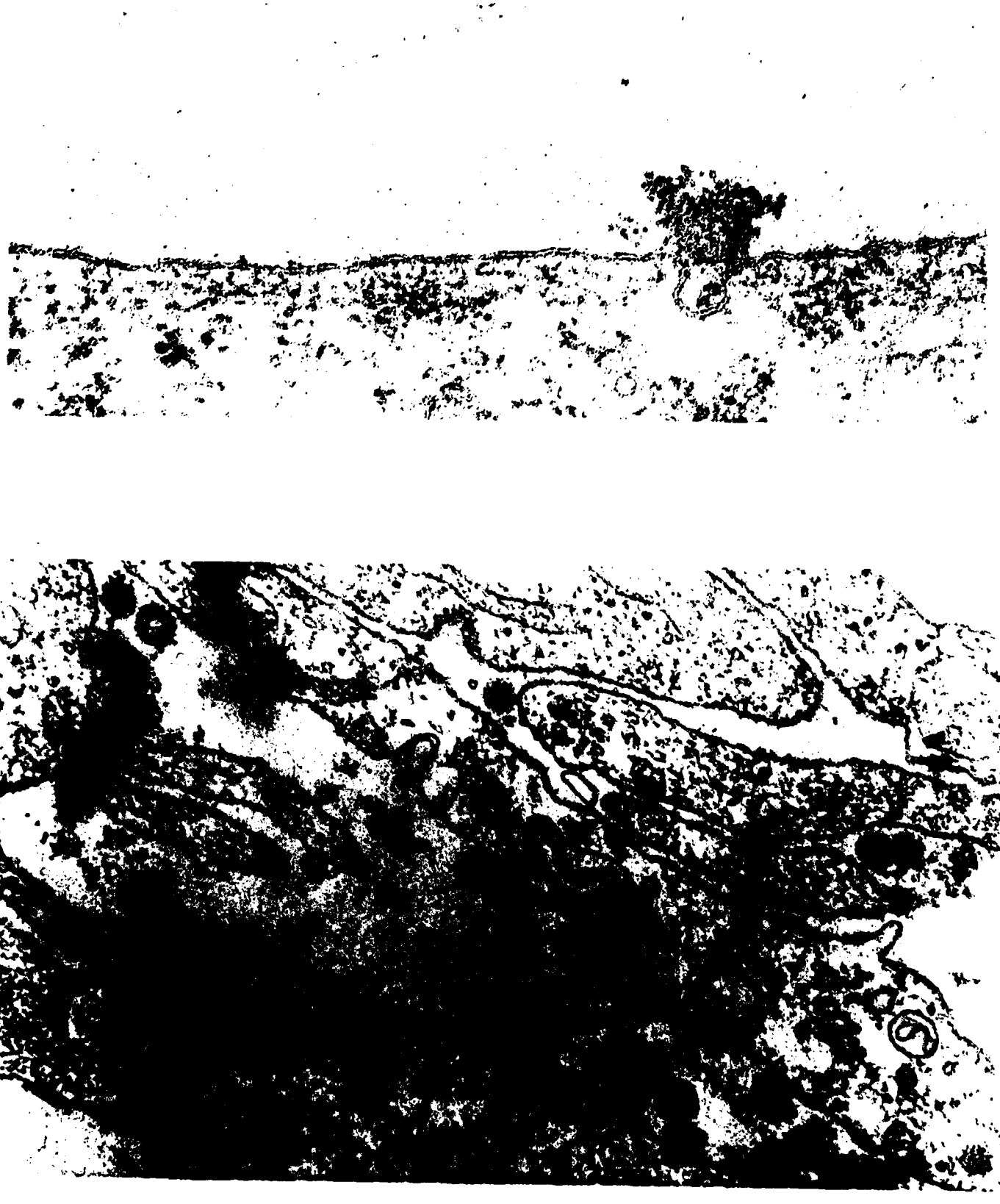


FIGURE 18a

INDIRECT FERRITIN-TAGGED ANTIBODY LABELLING OF KARIMBAD VIRUS-INFECTED CELLS

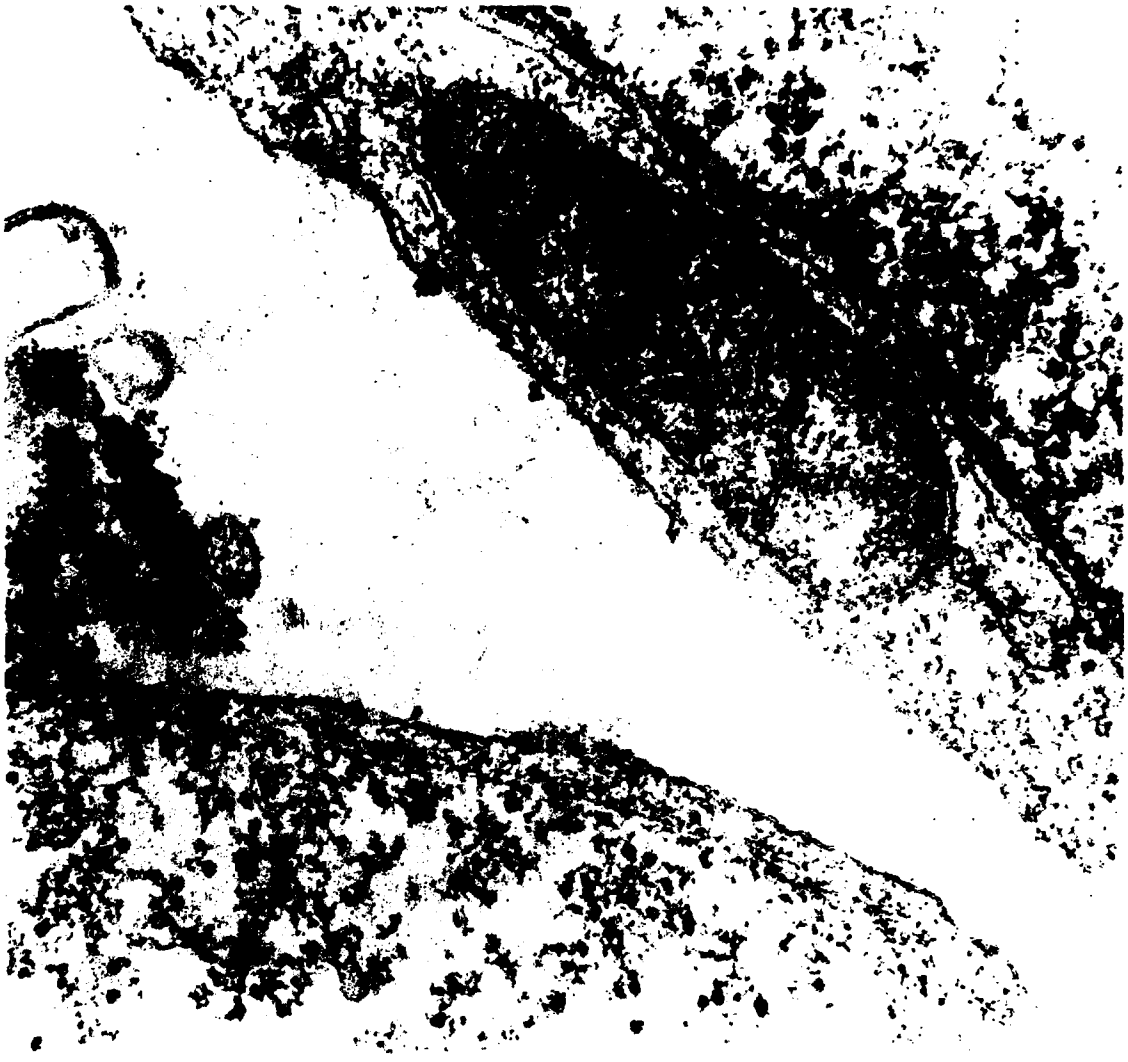


FIGURE 18b

INDIRECT FERRITIN-TAGGED ANTIBODY LABELLING OF KARIMIBAD VIRUS-INFECTED CELLS

FIGURE 19

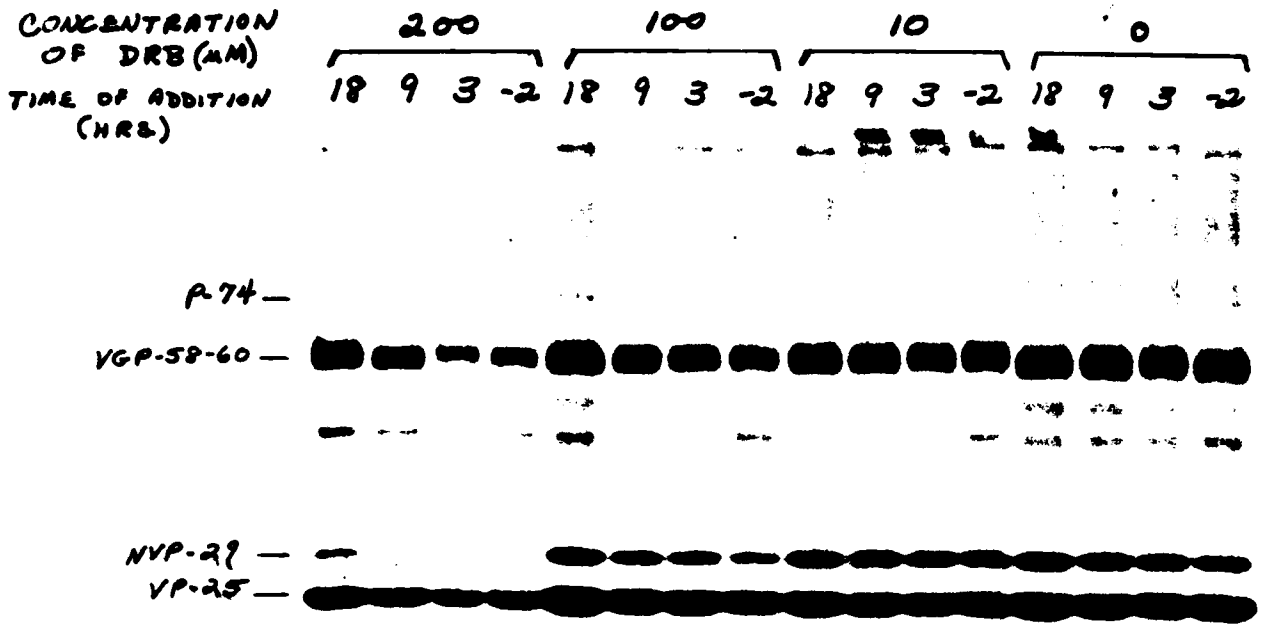


FIGURE 20

EFFECT OF DRB ON THE SYNTHESIS OF KARIMBAD VIRUS-SPECIFIC PROTEINS

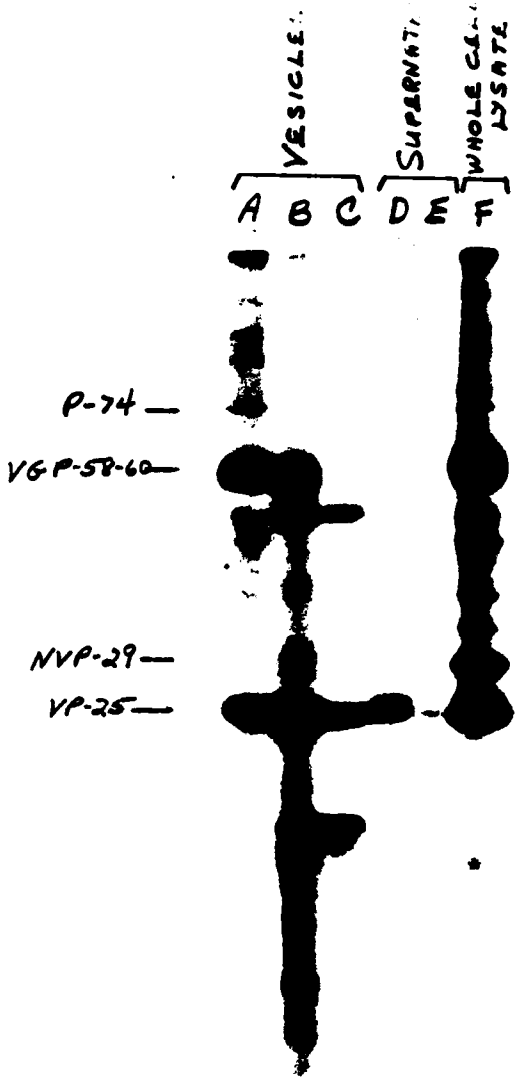
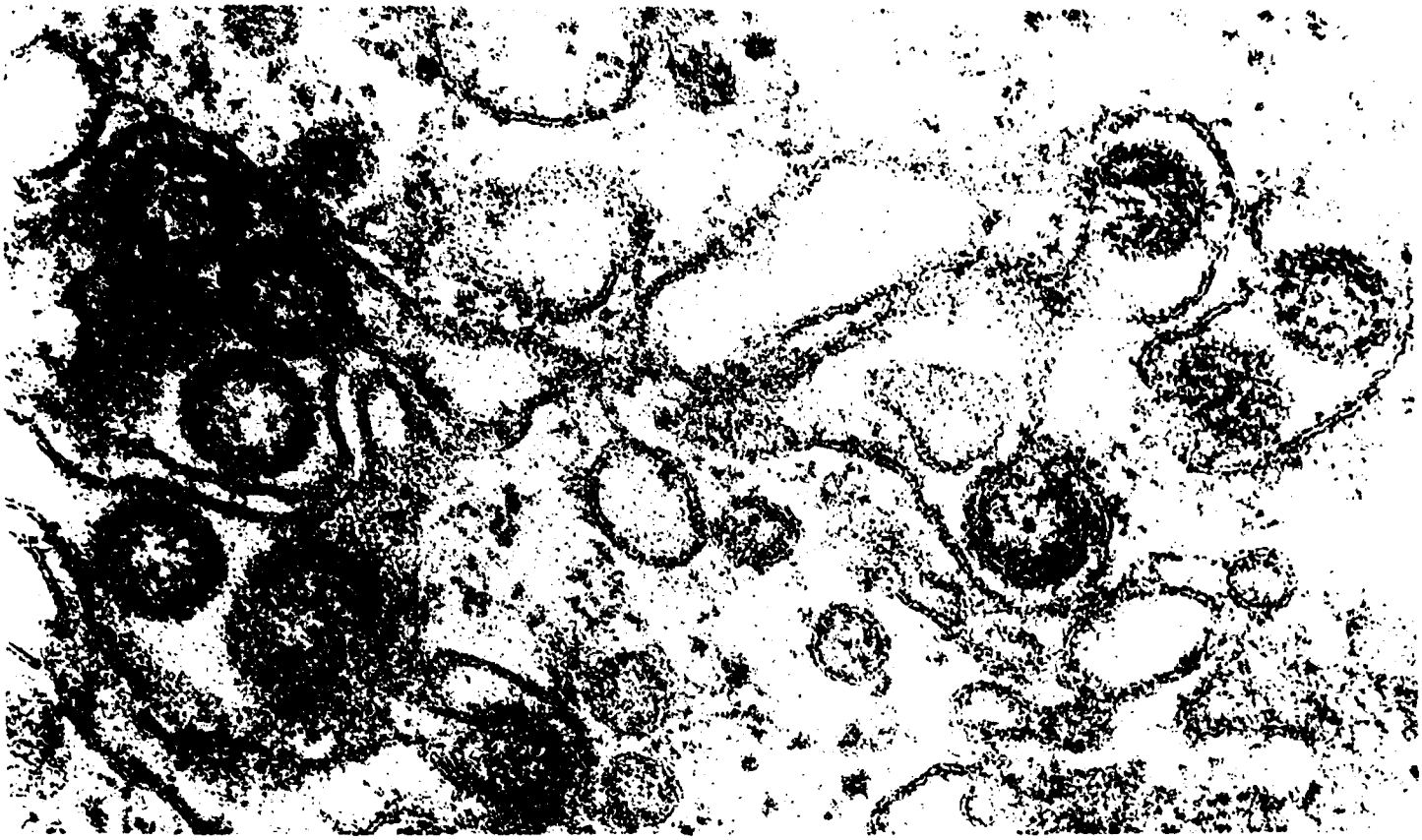


FIGURE 21

PROTEASE TREATMENT OF MEMBRANE VESICLES FROM KARIMBAD VIRUS-INFECTED CELLS

79 39 N/



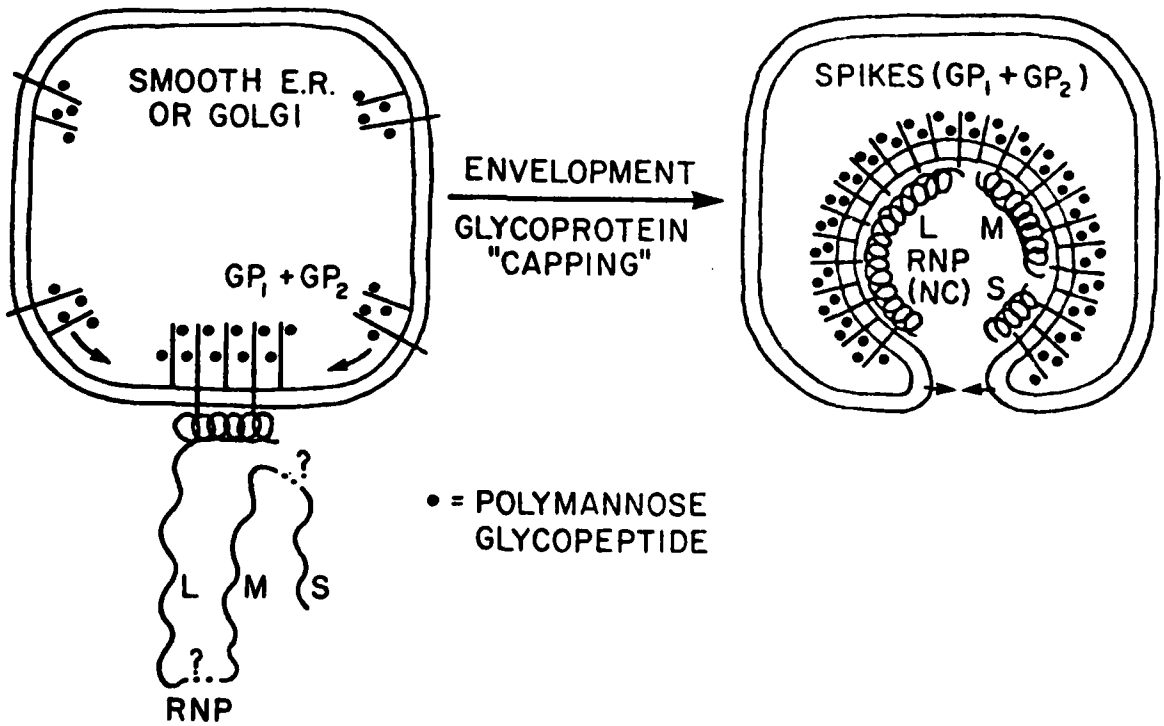

SMOOTH E.R.
OR GOLGI
GP1 + GP2
ENVELOPMENT
GLYCOPROTEIN
"CAPPING"
SPIKES (GP1 + GP2)
L
M
RNP
(NC)
S
• = POLYMANNOSE
GLYCOPEPTIDE
?
L
M
S
?
RNP

Figure 22

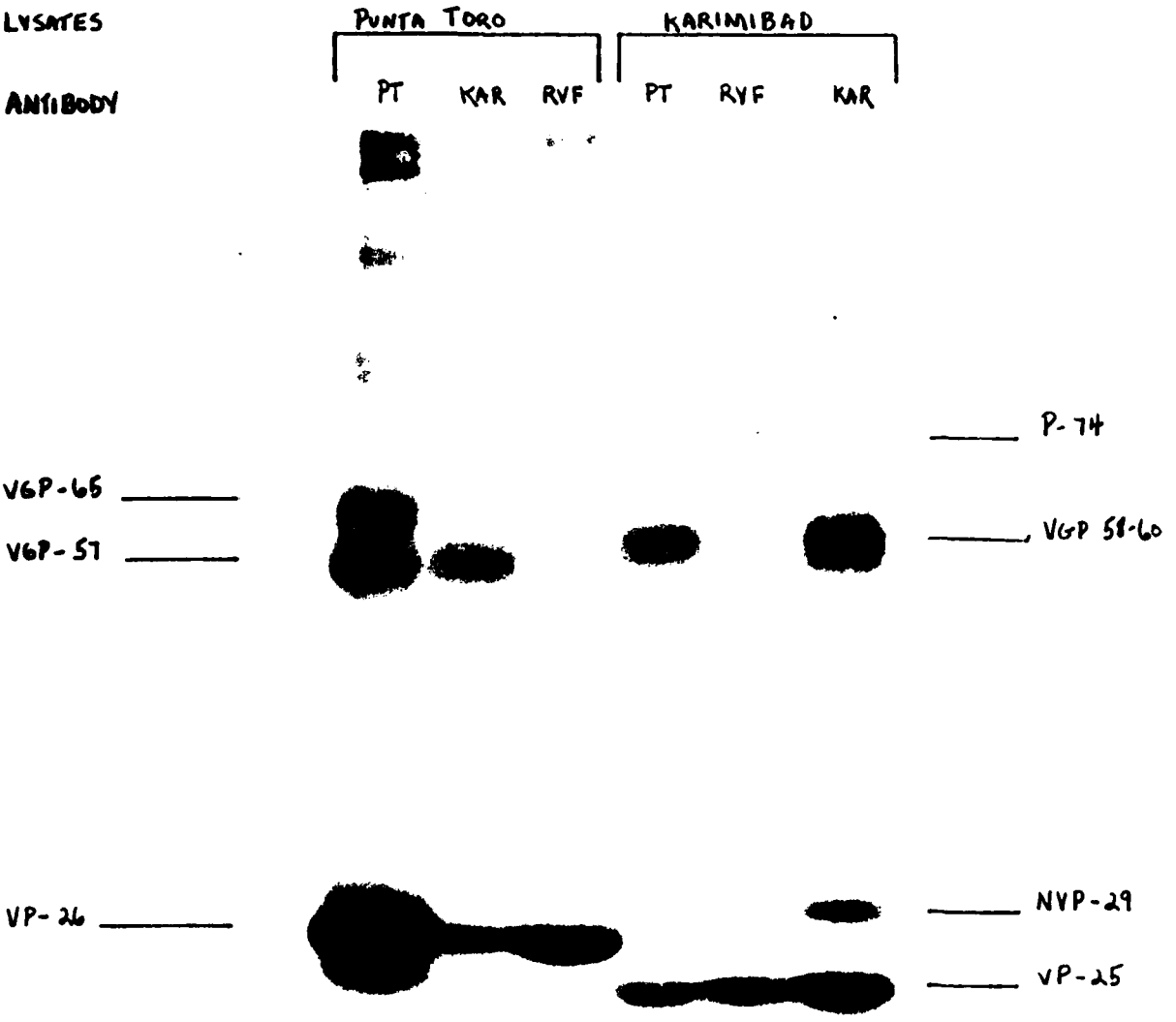


FIGURE 23

IMMUNOPRECIPITATION OF LYSATES FROM PUNTA TORO OR KARIMIBAD VIRUS-INFECTED CELLS WITH HOMOLOGOUS AND HETEROLOGOUS ANTIBODY.